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Genotypes of *Helicobacter pylori* isolates in Gambian children and adults

Thesis submitted to Open University, U.K. in fulfillment of the requirements of the Doctorate of Philosophy in the field of Sciences

2013

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Abstract

Helicobacter pylori is a globally important and genetically diverse gastric pathogen that infects most people in developing countries. Earlier reports indicated high prevalence of *H. pylori* colonization, but a low frequency of *H. pylori*-associated diseases in Africa but recent reviews have shown that gastroduodenal disease is actually common in Africa. Most detailed analyses of *H. pylori* have used strains from non-African countries, despite the high importance of Africa in the emergence and evolution of humans and their pathogens. There have been far fewer critical studies such as genotypes in association with gastric disease, population genetics and antimicrobial resistance and susceptibility of *H. pylori* strains from Africa.

It is with this background that the genotypes of *H. pylori* in association with gastroduodenal diseases, antibiotic susceptibility to commonly used drugs and population genetics from ethnic African adults and children in The Gambia were investigated.

In this study, it was found that the prevalence of *H. pylori* is high in dyspeptic patients in The Gambia and that many strains were of the putatively more virulent *cagA*⁺, *vacAs1* and *vacAm1* genotypes. There was a high prevalence of *cagA* positive strains in patients with overt gastric diseases than those with non-ulcerative dyspepsia; conversely, the co-existence of both *cagA*⁺ and *cagA*⁻ was found to be protective against the development of gastroduodenal disease.

Analyses of the sequenced data with the STRUCTURE software indicated that Gambian *H. pylori* strains were closely related to hspWAfrica than to strains from more distant African regions (hspSAfrica and hpNEAfrica) indicating common ancestral origin. Essentially no traces of European or North African ancestry were found despite Gambia's history of invasion and colonisation by peoples from these regions during the last millennium.

Antibiotic susceptibility tests have shown that Gambian strains were found to be highly sensitive to clarithromycin, erythromycin, tetracycline and amoxicillin but found that more than two-thirds of Gambian *H. pylori* strains were metronidazole resistant. These data indicate caution in use of metronidazole-based therapies in The Gambia.

This thesis provides a detailed initial description of a set of *H. pylori* isolates directly related to a geographically defined West African population. The data presented has answered some relevant questions on *H. pylori* virulent genes in association with gastro-duodenal diseases and antibiotic susceptibility providing a comprehensive basis for future studies.

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I would like to thank Allah, the Almighty for his guidance and light during the course of this study.

I would like to extend my gratitude to my mentors and supervisors:

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This study was funded by MRC unit, The Gambia and US National Institutes of Health (NIH) grants RO3-AI061308, R21-AI078237 and R21-AI088337.

Dedication

This work is dedicated to the loving memories of my Dad, Alh. Ebou Secka, my mother Safi Mbye, grandmother, Aji Fatou Sock, sister, Aji Adam Ebou Secka and uncle Alh. Musa Secka.

Statement of work performed

The biopsies used in this study were collected by gastroenterologists at the MRC unit, The Gambia and the taking of consent from study participants was performed by trained nurses. I was present in both processes outlined above.

I performed all of the following experiments and analyses at the MRC Unit, The Gambia:

- i. Bacterial culture, isolation and identification
- ii. PCR to detect *H. pylori* and *H. pylori* virulent genotypes
- iii. MLST of house-keeping genes and phylogenetic analyses
- iv. *rdxA* gene sequencing and analyses

I also performed the antibiotic susceptibility and resistant tests and *rdxA* transformation studies at Prof. Douglas E. Berg's laboratory, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA.

The STRUCTURE analysis presented in chapter 6 was performed by a Bioinformatician at the MRC Unit, The Gambia.

I performed all experiments presented in chapter 7 except for the RdxA structure modeling and identification of mutations of the *rdxA* gene which were performed at the Department of Biochemistry and Molecular and Cellular Biology, and Biocomputation and Complex Systems Physics Institute (BIFI)-Joint Unit BIFI-IQFR (CSIC), University of Zaragoza, Zaragoza, Spain.

Statistical support was received from a statistician for the data presented in chapter 4

Peer-reviewed publications from this thesis with myself as first Author

1. PCR-based genotyping of *Helicobacter pylori* of Gambian children and adults directly from biopsy specimens and bacterial cultures. **Secka O**, Antonio M, Tapgun M, Berg DE, Bottomley C, Thomas V, Walton R, Corrah T, Adegbola RA, Thomas JE. (2011). Gut Pathog 3: 5.
2. Mixed infection with *cagA* positive and *cagA* negative strains of *Helicobacter pylori* lowers disease burden in The Gambia. **Ousman Secka**, Martin Antonio, Douglas E. Berg, Mary Tapgun, Christian Bottomley, Vivat Thomas, Robert Walton, Tuman Corrah, Julian E. Thomas, Richard A. Adegbola. PLoS ONE. 2011; 6(11):e27954.
3. Antimicrobial susceptibility and resistance patterns among *Helicobacter pylori* strains from The Gambia, West Africa. **Secka O**, Berg DE, Antonio M, Corrah T, Tapgun M, Walton R, Thomas V, Galano JJ, Sancho J, Adegbola RA, Thomas JE. Antimicrob Agents Chemother. 2013; 57(3):1231-7. doi: 10.1128/AAC.00517-12.
4. Population Genetic Analyses of *Helicobacter pylori* Isolates from Gambian Adults and Children. **Ousman Secka**, Martin Antonio, Douglas E. Berg, Mary Tapgun, Robert Walton, Archibald Worwui, Vivat Thomas, Tuman Corrah, Julian E. Thomas, Richard A. Adegbola. PLoS ONE, In Press.

Abbreviations

-	Negative
+	Positive
+ve	Positive
-ve	Negative
µg	Microgram
µl	Microlitre
¹³ C	Non-radioactive carbon-13
¹⁴ C	Radioactive carbon-14
A.D	After death of Christ
Amo	Amoxicillin
ATPase	Adenosine triphosphatase
babA	blood group antigen-binding adhesin
bp	Base pair
cagA	Cytotoxic-associated gene A
camR	Chloramphenicol resistant
camS	Chloramphenicol sensitive
CFR	Code of Federal Regulations
Cla	Clarithromycin
CLO	Campylobacter-like organism
CLS	Clinical Laboratory services
cm	Centimetre
CO ₂	Carbon dioxide
CRF	Case report form
CRR	Central River Region
Dept	Department
DMID	Department of Microbiology and Infectious Diseases
Dn	Non-synonymous
DNA	Deoxyribo nucleic acid
DOB	Date of Birth
Ds	synonymous
DU	Duodenal ulcer
dupA	Duodenal ulcer promoting gene
EOP	Efficiency of plating
Ery	Erythromycin
E-test	Epsilometer test
Fe	Iron
FlaA	Flagella gene A
frxa	Flavin oxireductase A
FMN	Flavin mononucleotide
FWA	Federal Wide Insurance
g	Gram
GBA	Greater Banjul Area
GC	Gastric cancer
GCLP	Good Clinical Laboratory Practice
GCP	Good Clinical Practice
GE	Gastric erosion

GU	Gastric ulcer
H&S	Health and Safety
H₂O	water
HCO₃	Hydrogen carbonate
HIV	Human Immuno-deficient virus
homA	<i>H. pylori</i> outer membrane protein A
homB	<i>H. pylori</i> outer membrane protein B
HpaA	<i>H. pylori</i> adhesin A
iceA	Induce with contact epithelium A
ICH	International Conference on Harmonization
ID	Identity
IDA	Iron deficiency anaemia
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IRB	Institutional Review Board
ISM	Independent Safety Monitor
kanR	Kanamycin resistant
kanS	Kanamycin sensitive
KatA	Catalase gene A
Kb	Kilo base
KDA	Kilo daltons
Km	Kilo metre
LRR	Lower River Region
LSHTM	London School of Hygiene and Tropical Medicine
m/s	Metre per second
MEGA5	Molecular Evolutionary genetic Analysis version 5
mg	Milligram
MgCl₂	Magnesium chloride
MIC	Minimum inhibitory concentration
ml	Millilitre
MLST	Multilocus Sequence Typing scheme
mm	millimetre
MRC	Medical Research Council Unit, The Gambia
Mtz	Metronidazole
MtzR	Metronidazole resistant
MtzS	Metronidazole sensitive
MVLTS	Multi virulent Locus Typing scheme
NAD	Nicotinamide adenine dinucleotide
NapA	N-ethylmaleimide-sensitive factor attachment protein
NAPDH	Nicotinamide adenine dinucleotide phosphate-oxidase
NB	Note back
NBR	North Bank Region
ng	Nanogram
NH₄	Ammonium
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NUD	Non-ulcerative disease

OIPA	outer inflammatory protein
OPD	Outpatient department
OU	Oesophageal ulcer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
PhD	Doctor of Philosophy
PI	Principal Investigator
RAPD	Randomly Amplified Polymorphic DNA
<i>rdxa</i>	Nitroreductase A
rpm	Revolution per minute
rpoB	RNA polymerase, beta subunit
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RUT	Rapid Urease Test
RVTH	Royal Victoria Teaching Hospital
sabA	sialic acid binding adhesin
SEN	State Enrolled Nurse
SOP	Standard Operating Procedure
Sr.	Sister
SRN	State Registered Nurse
ST	Sequence type
TBE	Tris/Borate/EDTA buffer solution
Tet	Tetracycline
TetR	Tetracycline resistant
TetS	Tetracycline sensitive
Th1	T helper cells 1
Th2	T helper cells 2
TNF	Tumour necrosis factor
TsaA	translation state array analysis
TV	Television
UBT	Urea Breath test
UK	United Kingdom
UNDP	United Nation Development Program
ureaA	Urease gene A
URR	Upper River Region
US\$	United States dollar
USA	United States of America
UV	Ultra violet
V	Volts
<i>vacA</i>	Vacuolating cytotoxin gene
Vs	Versus
WCR	West Coast Region

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Chapter 1.0 Literature Review

1.1 Introduction

This study was part of a *Helicobacter pylori* (*H. pylori*) project jointly sponsored by the National Institute of Health (NIH) of USA and the Medical Research Council (MRC) Unit, The Gambia, West Africa and was conducted in The Gambia in a population with a high early childhood incidence of *H. pylori* colonization. All subjects enrolled in this study were Gambians of West African ethnicity.

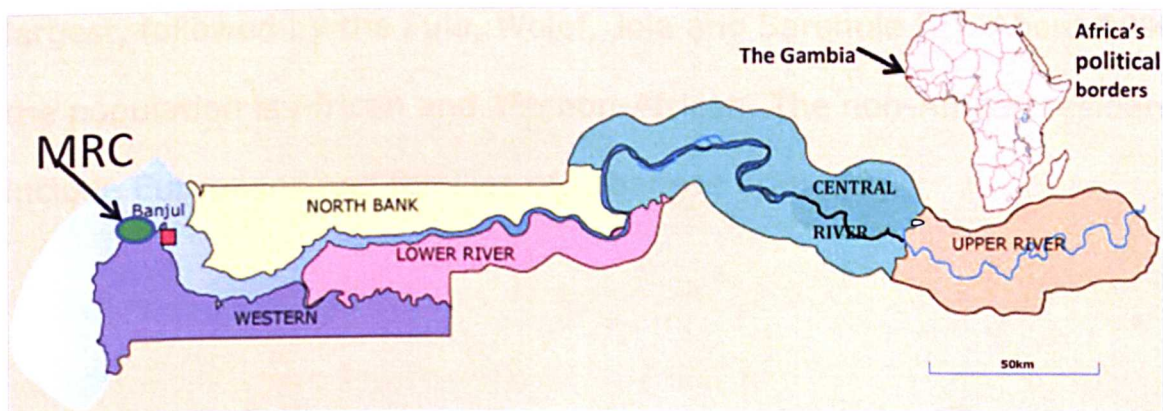
This was a descriptive study aimed at providing the first data to evaluate genotypes of *H. pylori* isolates obtained from study subjects living in The Gambia, genotypes and clinical outcome and to compare the differences with *H. pylori* genotypes from other countries and assess the population structure of the strains from this study population and their relatedness with strains from other geographically defined populations.

Taking part in this study was beneficial to the study subjects as it influenced treatment if associated diseases, such as peptic ulceration, gastric cancer or pre-cancerous lesions were detected. In these cases, *H. pylori* eradication therapy was offered.

1.2 The Gambia

The Gambia is a country in West Africa that has become a centre for tropical medical research, largely due to the MRC unit providing scientific and clinical facilities unique in West Africa. The country has some urban and semi-urban communities, but no major cities. It is the smallest country on mainland Africa, bordered to the north, east and south by Senegal, with a short coastline on the Atlantic Ocean and shares historical roots with many other West African nations.

Figure 1.1 Map of The Gambia showing the administrative regions



The country has an area of about 11 000 km², less than 48Km wide at its widest point and 338Km long. The country is divided into two halves by the Gambia River which flows through the country's centre and empties into the Atlantic Ocean. The Gambia is divided into five administrative regions (West Coast, North Bank, Lower River, Central River and Upper River regions) with 2 municipal areas –Banjul and Kanifing.

An agriculturally fertile country, its economy is dominated by farming, fishing, and tourism. About a third of the population lives below the internationally poverty line of US\$1.25 a day [1].

The climate of The Gambia is tropical. There is a hot and rainy season, normally from June until October, but from then until May there are cooler temperatures with less precipitation [2]. The Gambia is one of the most densely populated countries in the world with an estimated population of 2 million most of whom are Muslims (90%) and live in the rural villages (63%). A wide variety of ethnic groups live in the Gambia, each preserving its own language and traditions. The Mandinka tribe is the largest, followed by the Fula, Wolof, Jola and Sarahule [2]. About 99% of the population is African and 1% non-African. The non-African residents include Europeans and families of Lebanese origin [2].

The Gambia's first contact with Arabians was about the 9th century AD during the trans-Saharan trade, when Arab merchants and scholars established trade routes in West Africa to trade in slaves, gold and ivory. Contacts with Europeans, which was mainly in trade, began in the 15th century with the Portuguese and French, which turned into predominantly slave trade. In the 17th Century, The Gambia was colonised by the British and gained full independence from Britain in 1965.

1.3 Human gut Microbiota

The human body is inhabited by a large number of micro-organisms including bacteria which evolve throughout the life of the individual and play crucial roles in the maintenance of health but also contribute to the development of disease. The human gut of an average healthy adult individual contains about 100 trillion bacterial cells and outnumbers the human cells by 10 to 1 [3] and it is estimated that 500 to 1000 species of bacteria live in the human gut as normal gut microbiota [4].

Colonisation and composition of the human gut begins during birth upon passage through the birth canal [4] and thereafter influenced by other factors such as environment, infant diet, hospitalization, antibiotics and kinship [4-7]. In addition, host genetic factors also play an important role in the composition of the gut microbiota [4,5]. The normal gut microbiota in healthy individuals helps maintain the health of the host. However, the overgrowth of these micro-organisms which can lead to disease state is continuously checked and a balance maintained by the host immune system. The gut macrophages, T cells, B cells and secretory IgA maintain a proper immune response to the gut resident microbiota and invading pathogens [4,6] to prevent or control disease. In addition, the integrity of the gut mucosal barrier, gastric acidity and peristalsis normally control bacterial colonization of the human stomach thereby prevent disease.

1.4 Imbalances of the Gut and disease

Studies have revealed that deviations from the “normal gut microbiota” due to intrusion by antibiotics or invading pathogens leads to disruption of the fine balance between host and resident microbiota which may lead to disease states [8]. The consequent immune response of the host towards the invading pathogen may decrease the viability of resident microbiota allowing the invading pathogen to occupy the vacated niche and establish itself [9-11]. The proteobacteria which includes *H. pylori* have utilized these hosts’ responses effectively to successfully colonize the human gut and initiate disease processes [4].

1.5 Historical background

Contemporary scientific research has shown that the bacterium now known as *H. pylori* was recently rediscovered in 1982 [12]. This bacterium was originally referred to as *Campylobacter pyloridis* because of its similarity to *Campylobacter*. However, in 1989, flagellum morphology, fatty acid content and phylogenetic analysis using the 16s *rRNA* gene led to new evidence that this bacterium does not belong to *Campylobacter* [13] but rather in a different group within the Gram negative bacteria. Thus, the bacterium was placed in its own genus *Helicobacter* [13].

The first description of *H. pylori* dates back to the 19th century, when two German scientists called Bottcher and Letulle found spiral bacteria in the

lining of the human stomach [14]. In 1889, Jaworski found spiral-shaped bacteria, which he called *Vibrio rugula*, in gastric washings [14] and in 1892, the Italian researcher Giulio Bizzozzero identified spiral-shaped bacteria in the gastric mucosa of dogs [14]. The identified spiral-shaped bacteria could not be grown in conventional culture media and so these early studies did not have much impact and were eventually forgotten. In the 20th century, however, interest in this bacterium was reactivated when in 1982; Warren inoculated gastric biopsies on a non-selective chocolate agar. After two days incubation in micro-aerobic atmosphere, there was no visible colonial growth on the media plates. The plates were however unintentionally left for six days, and fortuitously there was visible bacterial growth of what is now known as *H. pylori* on the plates [13]. Robin Warren and Barry Marshall established the relationship of *H. pylori* with gastritis after they performed self inoculation with *H. pylori* and consequently developed symptomatic gastritis shortly after ingestion of the bacteria.

To date about twenty species have been identified infecting a varying number of hosts ranging from human to poultry [15]. The ecological niche of the species *H. pylori* in man is the gastroduodenal mucosa. However, *H. pylori* has been isolated from dental plaque, faeces and blood [16-19]. There is also evidence that *H. pylori* is found in water and the environment, although this has only been confirmed by molecular methods such as PCR and not by culture [20].

1.6 Detection of *Helicobacter pylori*

There are several methods that are available for the detection of infections caused by *H. pylori*. These methods can be grouped into invasive, semi-invasive and non-invasive and the choice of test largely depends on clinical status, availability and affordability. The objective for all the methods employed for diagnosis is to demonstrate that the *H. pylori* bacterium is present in the upper gastro-intestinal tract. In recent times, because *H. pylori* is a fastidious organism and difficult to culture, the approach has focused on detection of specific DNA sequences instead of culturing the bacterium and this has increased sensitivity.

1.6.1 Invasive methods

1.6.1.1 Upper gastric endoscopy

Upper gastric endoscopy is regarded as the gold standard and reference method used for the detection of *H. pylori* and diagnosing problems and diseases in the upper gastric tract associated with *H. pylori* infection such as gastric and duodenal ulcers, stomach cancers and gastritis. The procedure involves the examination of the oesophagus (gullet), stomach and duodenum (the first section of the small intestine) with a long flexible tube with a light source and a video camera attached. The images captured are viewed on a television (TV) monitor.

1.6.1.2 Biopsies for culture and Rapid Urease Test (RUT)

Gastric tissue is removed from the gastric mucosa by employing forceps passed through the operating tube of the endoscope to the gastric wall.

Biopsies are taken either from the body and/or antral part of the stomach.

1.6.1.3 Culture

The biopsies collected during endoscopy are directly inoculated on appropriate media which is incubated at 37°C in micro-aerobic atmosphere and examined for growth between 4-7 days [21]. The method is specific but sensitivity is low due to the fastidious nature of the organism [22].

1.6.1.4 Rapid Urease Test (RUT)

This test, also known as Campylobacter-Like Organism (CLO) test, utilizes the ability of *H. pylori* to produce large quantities of urease [23]. The biopsy obtained during endoscopy is inoculated into a medium that contains urea and phenol red indicator. If *H. pylori* is present in the tissue, the urease produced by the organism hydrolyzes urea to form carbon dioxide and ammonia thereby creating an alkali environment turning the medium from yellow to pink within one minute [24,25]. The sensitivity and specificity of the test is high [25,26]. The test is now commercially available; it is accurate and relatively cheap [26].

1.6.1.5 Histology

This method requires endoscopy and the collection of gastric biopsies. The formalin fixed or frozen biopsy samples are stained using Haematoxylin-Eosin, Giemsa, Silver stain or antibody-immuno stain and examined for the presence of *H. pylori* [27-30]. Although some staining techniques may be unreliable [31], sensitivities of up to 98% have been reported [30]. This technique requires expertise, and may take up to five days or more after sampling to obtain a result. However, a marked advantage of this method is that gastritis, intestinal metaplasia and atrophy can be accurately [32] assessed and confirmed.

Upper gastric endoscopy is an invasive and costly technique that causes discomfort to patients. In addition, although sterilization techniques of scopes have improved greatly, the risk of contamination by HIV and Hepatitis C viruses still remain [29]. Other disadvantages of this technique are that it only explores a small part of the stomach which can lead to sampling errors and only available in specialist endoscopy units which limits its use in developing countries and field studies. Thus, numerous non-invasive methods have been developed and evaluated over the years.

1.6.2 Semi-Invasive methods

1.6.2.1 Serological test

The method involves the use of venous or peripheral blood to measure IgG antibodies in response to *H. pylori* infection. Several methods have been described including the Enzyme-Linked Immunosorbent Assay (ELISA) [33,34] and commercially available Latex test kits. The tests are generally simple to perform, reproducible and cheap [35] but the sensitivity is low. The serological methods are very useful for epidemiological [36] and retrospective studies but cannot be used for the confirmation of successful treatment [37].

1.6.3 Non-Invasive methods

1.6.3.1 Urea Breath Test (UBT)

The UBT is based on the ability of *H. pylori* to breakdown urea into ammonia and carbon dioxide. A radioactive labelled carbon capsule containing urea (^{14}C) or a non-radioactive isotope (^{13}C) is swallowed by the patient. If *H. pylori* is present in the stomach, the urea is hydrolyzed into radioactive labelled carbon dioxide (CO_2) and ammonia. The CO_2 is absorbed in the blood and released in the lungs where it is exhaled in the breath. The extracted air is collected and measured. ^{14}C is a beta-emitting radioisotope and can be detected using liquid scintillation counting whilst ^{13}C is a stable, non-radioactive isotope that is measured using a mass spectrometer. The test is highly accurate, specific, sensitive and painless [23,38-40] and suitable for use in epidemiological studies including

children. However false negative results have been reported in cases where the patient is receiving acid suppression medications and/or is suffering from active bleeding [41-43] and also requires equipment which is routinely unavailable in most laboratories especially in resource poor countries. However, the test can be used to demonstrate that *H. pylori* has been eradicated after successful treatment with antibiotics [44].

1.6.3.2 Stool culture

There is continued evidence that *H. pylori* are transmitted through the oral-faecal route [45]. Although, this remains largely speculative due to the difficulty of successfully culturing the organism from stool. Several studies [17,46,47] have successfully isolated *H. pylori* from faeces albeit with great difficulty largely due to the presence of gut microbiota, susceptibility of the bacterium to bile salts and the fastidious nature of the organism [29]. These difficulties have resulted in the development of molecular and antigen based methods for the detection of *H. pylori* in stool.

1.6.3.3 Stool Antigen test

Several commercial methods are now available for the detection of *H. pylori* antigen in stool [48-50]. These antigen-based tests are highly sensitive and specific [51,52], simple to perform, cheap and do not require specialized equipment and/or personnel. They are also very useful

in large scale epidemiological studies especially in children and can also be used to evaluate success in *H. pylori* eradication therapy [53].

1.6.3.4 Urine antibody test

A rapid urine test (RAPIRUN^R) is a commercial method used to detect anti-*H. pylori* IgG. The test was found to be highly specific and sensitive and is useful for *H. pylori* screening [54]. However most of these studies have been done on Asian strains and therefore the test kits should be evaluated with local strains.

1.6.3.5 PCR of stool

In recent times, because *H. pylori* is a fastidious organism and difficult to culture, the approach has focused on detection of specific DNA sequences instead of culturing the bacterium and this has increased sensitivity. These DNA based methods (PCR) have been successfully used to detect *H. pylori* from stool and has shown acceptable sensitivity and specificity [55] despite the presence of inhibitors [56,57] and also *H. pylori* degradation in the intestine [58].

1.7 Epidemiology

H. pylori is endemic in human populations colonizing the human gastric mucosa leading to the development of gastro-duodenal diseases in adult life, usually after years of chronic infection [59,60]. Prevalence of about

20% has been reported in developed countries and over 80% in developing countries including Africa [19,61-67].

Once established, colonization usually persists for a very long time unless treated by antibiotics [68]. Chronic *H. pylori* infection is one of the strongest key factors in the etiology of various gastrointestinal diseases, ranging from chronic active gastritis without clinical symptoms to gastric and duodenal ulceration and gastric adenocarcinoma [69-74].

The high prevalence in developing countries do not parallel the development of *H. pylori*-associated diseases as peptic ulcers and gastric carcinomas were reportedly rare in individuals from these countries particularly in Africa [19,62,67,75] compared to those from Europe and USA [76-78]. This observation, which has been termed "The African Enigma", remains largely unexplained. It is however, believed that geographical variations in pathogenic determinants between isolates of *H. pylori* and variations in stomach ecology, host immune mechanisms and physiology between human populations may be very important factors [47].

However, recent reviews and studies have shown that, the prevalence of gastric diseases such as gastric cancers, gastric and duodenal diseases in Africa are indeed comparable with those in Europe [79,80]. The "Africa Enigma" may have been due to poor maintenance of cancer registries,

shorter life expectancy compared to developed countries, inaccessibility to competent health facilities to accurately diagnose these diseases and poor study designs.

1.7.1 *H. pylori* genotypes in children

H. pylori are acquired very early in life particularly in Africa where up to 96% of children are infected by the age of five years [19,65]. The studies that have so far characterized child isolates have also shown marked geographical differences of genotypes. For example, studies in Brazil, Slovenia, Colombia, North America, Korea and Japan [81-86] have shown that between 64-75% of all child isolates carried the virulent *s1* allele of *vacA* gene. 82% and 94 % of Korean isolates [84] carried the *m1* allele of *vacA* gene and *cagA* gene respectively which is similar to figures reported in Japan [81]. Similarly in Brazil [82], the majority of child isolates were *m1* allele positive. This may be a contributing factor to the high risk of gastric carcinoma in adult life in these populations [39,86]. In contrast, a significant number of children carried the less virulent genes of *cagA* and *vacA* in Portugal [87] than isolates from adults. This is similar to figures reported in Israel [88] where only about 25% of children carried the virulent *cagA* gene and 65% of the avirulent *vacA* alleles of *s2m2*.

1.7.2 Transmission

The transmission of *H. pylori* is poorly understood because of the difficulties of obtaining samples for large epidemiological transmission

studies. It is believed that transmission of *H. pylori* occurs mainly at an early stage in life and mainly between siblings [89]. Several studies have considered close contact between individuals through vomitus, saliva [90,91] and also direct contact [92] as important routes for transmission. Individuals can also be infected through the faecal-oral route by ingesting contaminated water and food [90,92]. In recent studies in China and Iran, similar *H. pylori* genotypes were found in both the saliva and stomach of patients indicating that saliva can be both a transmitting and re-infecting vector [93,94]. However, a study in Europe found that oropharyngeal infection seem to be independent to gastric infection [95]. It has been observed in some studies [96] that the house fly is a vector for the transmission of *H. pylori*. However, in Gambian villages where there is a high prevalence of *H. pylori*, the house fly is neither an important reservoir of infection nor a major route of transmission [97].

The only proven route of transmission is the hospital acquired infection during endoscopy. This iatrogenic transmission can occur when the endoscopy is not adequately disinfected [98,99]. Generally low sanitation, low social class and overcrowding are important risk factors for the transmission of this organism particularly in developing countries.

The prevalence of *H. pylori* infection in industrialized countries has declined dramatically during the last century [100], probably due to improvements in hygiene and sanitation. In these societies, the pattern of

transmission is predominantly intrafamilial [89,101] (although community transmission is frequent in developing countries), and there is also generally low risk of infection in adulthood.

1.8 *H. pylori* pathogenesis

H. pylori is one of the most successful human pathogens and over half of the world's population is colonized with this Gram-negative bacterium with prevalence of over 80% in many developing countries including The Gambia [19,62,63,102], typically starting in infancy [19,102,103] and lasting for life [104]. *H. pylori* isolates possess substantial genotypic diversity, which engenders differential host inflammatory responses that influence pathologic outcome. The majority of people infected with *H. pylori* remain asymptomatic; only about 20% eventually proceed to develop severe *H. pylori* associated diseases. Distinct *H. pylori* genotypes have been linked to the risk of gastroduodenal diseases. These include the *vacA*, *cagA* and *iceA* genes. The *vacA* mid region shows a marked geographic variation. For example, the majority of European *H. pylori* strains carry the *m1a* allele, whereas, Asian and Indian *H. pylori* strains carry the *m1b* and *m1c* alleles respectively [21].

Most *H. pylori* strains from patients with gastro-duodenal disease from industrialized countries carry the *cagPAI*, whilst the majority of strains from subjects with *H. pylori* colonization lack *cagPAI* [105]. For example, 60% of *H. pylori* strains isolated from patients with gastric disease in

industrialized countries carry the *cagPAI* [106]. However, almost all Asian *H. pylori* strains carry the *cagPAI* and the toxigenic “*s1*”-type alleles of *vacA* independent of disease [21]. The majority of *cag*-positive strains also carry the *s1* allele of *vacA* [107-109], whereas majority of *cag*-negative strains contain the non-toxigenic *s2* allele.

The *iceA* gene which consists of two alleles; *iceA1* and *iceA2* [105] have been associated with disease outcome. For example, *iceA1* isolates have been seen significantly more often in patients with peptic ulcer diseases in Turkey [110,111] and gastric carcinoma in South African patients [112] whereas the *iceA2* genotype was mostly reported in patients with gastritis [110,112] in a study in South Africa.

Most studies [21,105,113] that investigate this diversity focused on bacterial factors such as *cagA* and *vacA* genes. There have been conflicting and inconsistent reports as some countries with high prevalence of *H. pylori* carrying the *cagA* and *vacA* genes do not parallel disease outcome [21,114-116]. Further, a study in Colombia [117] has found that there is a 25-fold increase in gastric cancer rate in the mountain regions as compared to coastal areas despite similarities (~90%) in *H. pylori* prevalences. This therefore suggests that apart from bacterial factors, there are other important determinants of the risk of overt disease such as human genetic and physiology, nutrition and

environmental factors which significantly contribute to *H. pylori* associated disease outcome [118,119].

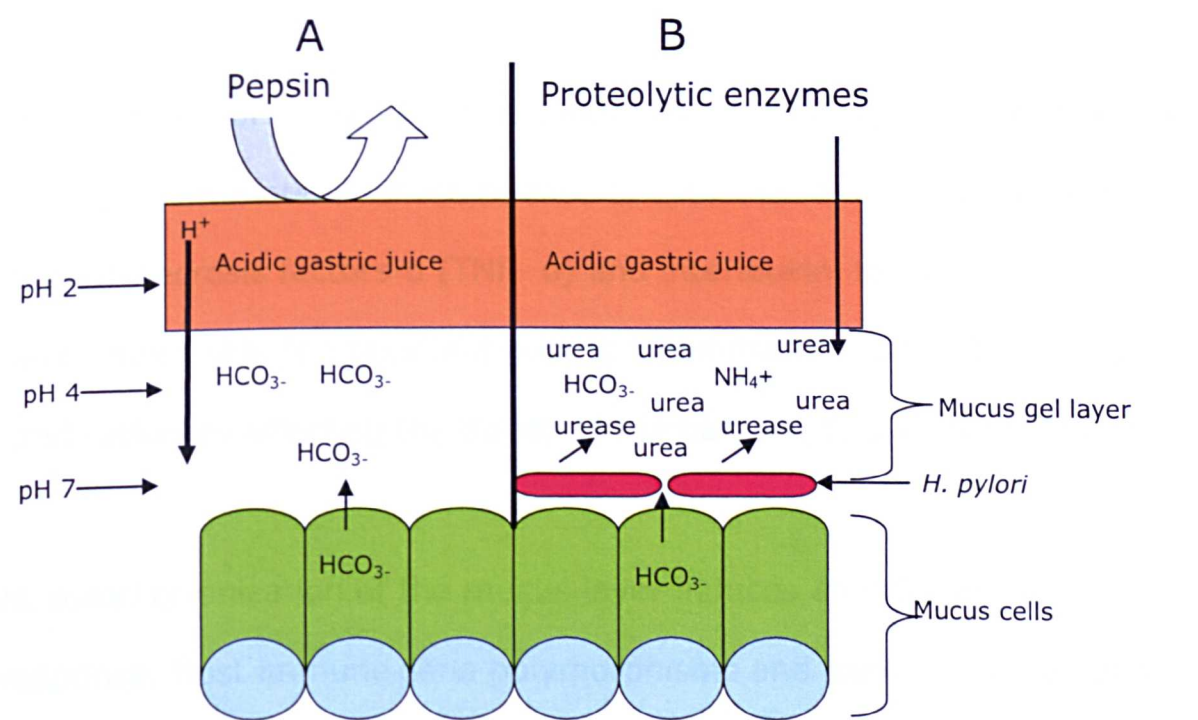
It has not been clearly established as to how the presence of *H. pylori* leads to gastric and duodenal ulcers, but disruption of gastric and duodenal mucosal integrity seems to involve a complex interaction between the host and pathogen. The complex interaction between bacterial, host genetic and environmental factors lead to different clinical outcomes of *H. pylori* infection in different geographic regions of the world. *H. pylori* have developed effective ways of evading the host immune response during colonisation and establish persistent infection [120,121].

1.8.1 Urease gene

A thick layer of mucus that covers the stomach lining protects the stomach from its own gastric juice. *H. pylori* take advantage of this by living in the mucus lining. Once *H. pylori* is in the mucus layer, it is able to fight the harsh condition of the stomach with copious production of the enzyme urease. The urease which has two subunits (ureaseA -3KDA- and ureaseB -62KDA) [122] is found in both the cytoplasm and cell membrane of the organism and is produced in large quantities both in vivo and in vitro [99]. The urease gene plays a key role in the colonization, pathogenicity, motility and survival of this organism. The urease genes are conserved in most strains of *H. pylori* and are used for the detection

of *H. pylori* in biological specimens [123]. After being ingested, *H. pylori* produce copious amounts of urease which neutralises the acidic contents of the stomach to an optimal pH for survival [120,124]. The urease hydrolyzes urea to form carbon dioxide and ammonia as a by-product thereby neutralizing the excess acid found in the stomach, creating a more basic pH between 5.5 to 8.5 (figure 1.2) and conducive environment for survival [125]. The strong bases protect *H. pylori* from the acid in the stomach [126]. The large quantities of ammonia and ammonia by-products produced such as protease and phospholipases (enzymes that break down proteins and hydrolyze phospholipids into fatty acids and other lipophilic substances respectively) chloramine [127] have also been shown to have cytotoxic effects [127].

Figure 1.2 Schematic representation of urease production and hydrolysis in the stomach.



A=without *H. pylori* colonisation, there is a normal pH gradient without damage to the gastric mucosal barrier.
B= in the presence of *H. pylori*, the urease produced by the organism hydrolyzes urea to form carbon dioxide, ammonia and proteolytic enzymes that damage intestinal mucosal barrier.

Further, the urease activity that reduces gastric acidity, the shape, mobility, the action of the bacterium's polar flagella that counteracts peristalsis [128], and mucus degrading ability [129] of *H. pylori* assist the bacteria to invade the mucus layer of the stomach and bind to the inner surface of the gastric epithelial cells with the help of many adhesions including BabA, OipA, SabA etc [120].

1.8.2 Host Factors

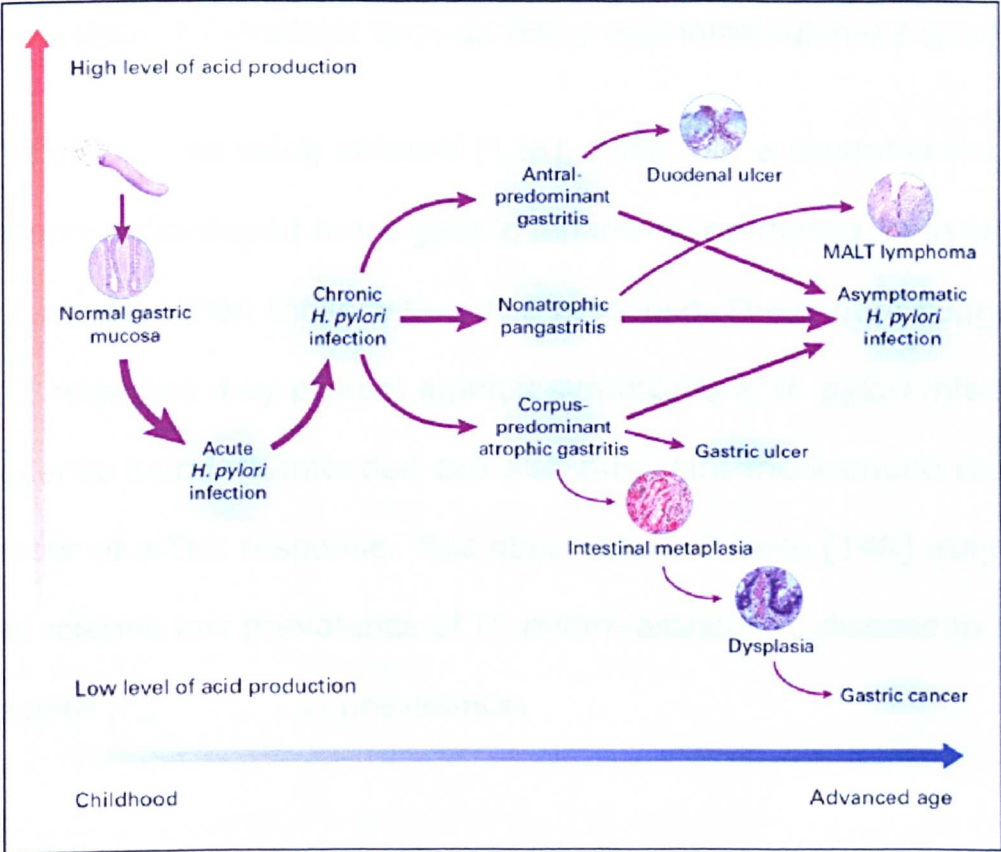
Several studies have shown that apart from bacterial factors, host genetic polymorphisms are also very important in determining *H. pylori* associated diseases. In general the over expression of the pro-inflammatory cytokines such as IL-1 β , IL-8, increased TNF- α and gastrin were shown to be associated with the development gastritis and gastric cancer [118].

The presence of *H. pylori* in the gastric mucosa induces both active and chronic inflammation via the release of cytokines such as interleukin-8, tumour-necrosis factors- α (TNF- α) and interleukin-1 β [130]. Studies have shown that the resultant gastric inflammation can lead to *H. pylori* eradication by affecting the ability of the bacteria to survive [131].

H. pylori colonization of the mucus layer induces an inflammatory response. Host immune gene polymorphisms and gastric acid secretion largely determine the bacterium's ability to colonize a specific gastric

niche. The severity of this inflammation underlies the type of gastritis (type B or type AB) [130,132] and *H. pylori* clinical diseases such as duodenal and gastric ulcers [133]. It has been shown that the amount of acid produced in the stomach correlates with the type of ulcer [132]. The large amount of acid produced by some people may confine *H. pylori* in the antrum and the subsequent inflammatory response may induce the production of gastric juice which stimulates the corpus to secrete more acid [134,135]. The increased acid load damages the duodenum leading to duodenal ulcers. In contrast, normal or low acid production in some people allows *H. pylori* to survive in the body of the stomach. The chronic infection leads to further reduction of acid production which leads to atrophy of the stomach lining, gastric ulcer and gastric cancer as illustrated in figure 1.3 which shows that disease development takes decades after colonisation [124].

Figure 1.3 Development of disease following *H. pylori* infection



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This observation is consistent with findings in humans [132] and also in mouse models [136,137] which suggest that susceptibility to *H. pylori* infection and disease development may partly be dependent on host genetic factors.

1.8.3 Cellular responses

H. pylori infection provokes an acquired immune response with the production of antibodies and either a Th1 or Th2 response [138]. During active disease, *H. pylori* induces a Th1-mediated pro-inflammatory

response that recruits CD4⁺ T cells resulting in the increased local production of cytokines such as IFN- γ and interleukin-12 [139].

In experiments using animals [138], mice with a predominant Th1 response developed more gastric inflammation during *H. pylori* colonization than those with a Th2 response. These data suggest that a Th2 response may protect against symptomatic *H. pylori* infection. Endemic helminth infection can also modulate the immune response in favour of a Th2 response. This observation in mice [140] may also explain the relative low prevalence of *H. pylori*-associated disease in Africa despite high *H. pylori* prevalence.

1.8.4 *H. pylori* and non-gastroduodenal disease

H. pylori colonisation also increases the risk of infection by diarrheal pathogens [141], iron deficiency [142] infant malnutrition and growth faltering in low income societies [143,144].

1.8.4.1 Iron-deficient anaemia

Iron deficiency anaemia (IDA), which is one of the most common nutritional deficiencies in the world, has been associated with *H. pylori* infection. Almost all pathogenic bacteria including *H. pylori* require iron (Fe) for metabolism and growth. *H. pylori* could cause iron deficiency indirectly by suppressing gastric acid secretion, thereby reducing solubility and uptake of dietary iron. There is also evidence obtained with a mouse

infection model suggesting that *H. pylori* successfully competes for dietary iron when the diet is iron poor [145]. Other potential explanations for an association between iron deficiency and *H. pylori* include occult blood loss from gastric erosion. Thus, in addition to host and environmental factors, colonisation of the stomach by *H. pylori* may be involved in the development of IDA [146]. Several studies have shown a strong association between *H. pylori* infection and IDA [142,147,148] regardless of the presence or absence of peptic ulcer disease [149].

1.8.4.2 Diarrheal diseases

It is believed that low acid secretion (hypochlorhydria) as has been seen in Gambian children [150] and the loss of gastric acid barrier could increase susceptibility to gastric pathogens thereby increasing the risk of diarrhoea in *H. pylori* infected individuals. In a study in Peru [141], children infected with *H. pylori* had twice the risk of diarrhoeal disease in a year than uninfected children.

1.8.4.3 Malnutrition and growth faltering

Loss of gastric acid barrier and injury of the lining of the small intestine due to *H. pylori* infections may lead to damage to the intestinal mucosal barrier and poor absorption of nutrients which leads to malnutrition and growth faltering. In The Gambia where *H. pylori* colonisation in infancy is high [19,62], significant growth faltering and malnutrition was found in children with early *H. pylori* colonisation.

1.8.4.4 Diet and gastroduodenal disease

H. pylori infection is an established risk factor for the development of gastro-duodenal disease but the development of gastroduodenal disease is influenced by other factors. The incidence of gastric disease varies in different ethnic groups and geographic locations [75,151,152]. Among the factors implicated in this difference is diet, which is an important factor in the development of gastroduodenal disease. Animal and case-controlled studies in humans [153] have shown that the risk of gastric cancer may be increased with a high intake of salt, preserved foods and foods high in fat and low intake of fruits and vegetables. Diet high in fruit and vegetables was shown to be protective against gastric cancer in an urban Portuguese population and also in USA [154,155]. Further, a 25-fold increase in gastric cancer was observed in a population in South America [117] whose diet was predominantly starch-based when compared to those with sea food based diet. Studies have shown that Nickel is important in *H. pylori* survival. Nickel free diet has been shown to reduce urease activity and expose the bacterium to the gastric acid with a decrease of survival [156]. Similarly in Korea [157,158], a Korean pickled dish was shown to contain a strain of bacteria called *Lactobacilli plantarum* NO1. This bacterial strain was found to reduce the activity of urease found in *H. pylori* by 40-60% and consequently antagonised the ability of *H. pylori* to bind to human gastric cell lines [158]. Red ginseng from Korea, a natural herb and an extract of licochalcone A were shown to have properties that stops inflammatory carcinogenesis associated with *H.*

pylori infection thereby blocking the progression of *H. pylori* associated precancerous lesion respectively [157].

1.8.4.5 Benefits of *H. pylori* colonisation

Studies have shown that *H. pylori* have co-evolved with human kind [159,160] for more than 60 0000 years. There have been suggestions that since *H. pylori* has been with man for thousands of years, it must have possible benefits to mankind [161]. It has been reported in some studies that *H. pylori* colonisation may be beneficial [162,163] as disorders such as esophageal diseases, childhood-onset asthma and tuberculosis are less likely to occur in patients infected with *H. pylori* [162-164]

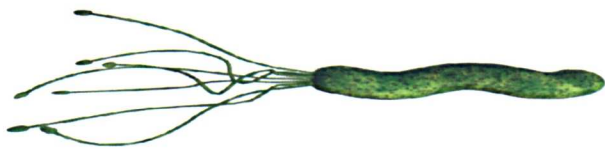
Several studies have reported an inverse relationship between *H. pylori* and Gastro esophageal reflux disease (GERD) [165]. GERD is a condition in which the stomach contents leak backwards from the stomach into the oesophagus. This action can irritate the oesophagus, causing heart burn and other symptoms. Despite the benefits shown in above studies, systemic studies at least in Western societies showed that there is no relationship between *H. pylori* colonisation and GERD [166].

1.9 Characteristics of *H. pylori*

H. pylori belonged to the family Helicobacteriaceae and it is spirally shaped as shown in figure 1.4. The genus *Helicobacter* is derived from

two Greek words "helix" which means "spiral" or "coil" and "bakterion" which means "small staff" [167]. The species name "*pylori*" also came from two Greek words such as "pyle" which means "gate" and "ourus" which means "keeper" [167], referring to the pylorus region of the stomach where *H. pylori* mainly resides.

Figure 1.4 3-D image of *H. pylori*



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1.9.1 Laboratory diagnoses

1.9.1.1 Cultural characteristics and growth requirements

H. pylori is a motile, non-spore forming bacteria. It is a fastidious organism and technically very difficult to grow and maintain as repeated culture leads to loss of viability [168]. Its successful growth on culture media is dependent on several factors such as temperature, oxygen stress, availability of nutrients [168], and pH between 5.5-8.5. There are several basal media such as Columbia agar [21], Brain Heart infusion agar [169,170], Brucella [22] and Mueller-Hinton agar [26] enriched with blood or blood products which have been described and used for the isolation of *H. pylori*. *H. pylori* grows well on Columbia agar supplemented with 10% laked or lysed horse blood and 1% vitox (Oxoid, Basingstoke, UK) which contains micro-nutrients such as vitamin B12, adenine, L-

cystine, cysteine, Nicotinamide adenine dinucleotide (NAD). It is a micro-aerophile and requires an atmosphere composed of 86% Nitrogen, 4% oxygen, 5% carbon dioxide and 5% hydrogen [171] to grow. After 3-7 days incubation at 35-37°C, on 10% Columbia-Blood agar, *H. pylori* colonies are grey, convex and translucent colonies of 1-2mm diameter. The morphology of this organism changes from a typical spiral or rod-shaped to coccoidal forms after 10 days of storage [168]. These changes are attributed to reduction in DNA, RNA and ATP quantities [168] as a result of unfavourable conditions such as nutrient starvation during prolonged incubation, exposure to antibiotics and extreme temperature and pH. These coccoidal forms are generally unculturable [15] but may be involved in transmission [172].

1.9.1.2 The use of gas generating kit

The atmosphere in which *H. pylori* is incubated is a critically important factor in the successful growth and isolation of the bacteria from clinical specimens. Micro-aerobic incubators are used primarily in the study and isolation of micro-aerophilic organisms such as *Campylobacter* species and *H. pylori* and other similar organisms. Here up to four gases - nitrogen, carbon dioxide, air and a 10% hydrogen/nitrogen mix - can be combined within safe and varying ratios to provide a specific atmosphere. These incubators however are expensive and not within reach of most laboratories in the developing world. Therefore, gas generating kits (GasPaks) have been used as a cheaper and more convenient alternative.

However, these GasPaks have been shown to provide a variable micro-aerobic atmosphere [29,173]. The generation of a suitable micro-aerobic atmosphere using GasPaks can take up to thirty minutes, during which time the stressed *H. pylori* becomes non-culturable as a result of the toxicity of high levels of oxygen.

1.9.1.3 Gram Stain appearance

The Gram reaction of *H. pylori* exhibits a marked degree of pleomorphism. It is helical, comma-shaped and sometimes straight Gram-Negative bacilli measuring between 0.5-1.0µm in diameter and 1.5-10µm in length [171].

1.9.1.4 Extracellular enzyme activities

H. pylori is mostly inactive in conventional biochemical reactions as it neither oxidize nor ferments carbohydrates. However, it is catalase and oxidase positive. *H. pylori* is also urease positive and this ureolytic activity is usually very rapid and strong.

1.10 Genomic features and genetic diversity of *H. pylori*

In recent years, the complete genome sequences of several *H. pylori* isolates have been published [174-177]. The genome (a circular DNA) is about 1.6 to 1.7 mega bases [177] with a low G+C content of about 32-44% and is relatively small when compared with other bacteria such as

Staphylococcus aureus [178] and *Neisseria gonorrhoeae* [179]. The genome contains some predicted genes that encode surface exposed and secreted proteins that are likely to be important in host-pathogen interactions [180].

A most striking feature of *H. pylori* as a species is its high genetic diversity: any 2 independent clinical isolates even from the same community differ on average by some 3% or more in base substitutions in essential housekeeping genes and 5% or more in gene content [181]. Individual isolates are almost always distinguishable from one another by random amplification of polymorphic DNA (RAPD)-PCR typing or focused DNA sequencing of 1 or 2 housekeeping genes [182]. This diversity has been ascribed to multiple factors: (i) frequent mutation throughout the genome [183]; (ii) ON-OFF switching of certain contingency genes due to frame shift mutations in repetitive sequence tracts [184]; (iii) extensive recombination between *H. pylori* lineages [185]; (iv) selection stemming from differences among humans in traits important to individual strains, which may be strongest immediately after colonization of a new host and again later, as host immune and inflammatory responses develop; (v) *H. pylori*'s preferential transmission within a family or local community [89,186], and (vi) the extraordinary chronicity of infection (frequently for decades), which creates a highly fragmented population structure, equivalent to island populations of classical higher organism population genetics [187]. Collectively, these features diminish the chance that few

genotypes might emerge ideally adapted for all human hosts. Rather many evolutionary trajectories may develop, a situation likened to “a rugged evolutionary landscape” [188].

1.10.1 *H. pylori* virulence genes

Amongst the genes known to play a role in disease causation are *cagA*, encoded within the *cag* pathogenicity island (*cagPAI*), the vacuolating cytotoxin, *vacA* and *iceA* gene (induced by contact with epithelium).

1.10.1.1 *cagA*

The *cagA* protein is produced by a majority of *H. pylori* strains [189].

Once *H. pylori* comes in contact with the stomach gastric epithelial cells, this toxin is injected into the cells of the stomach lining thereby altering the structure of the stomach cells and allowing the bacteria to attach themselves more readily leading to chronic inflammation. The cells undergo tyrosine phosphorylation [190,191] which eventually leads to morphological transformation [191]. Long-term exposure to the toxin causes chronic inflammation. However, not all strains of *H. pylori* carry the *cagA* gene; those that do are classified as *cagA*-positive.

The strains that do not produce this toxin (*cagA* negative) are believed to be less virulent. Most isolates from patients with gastro-duodenal diseases from industrialized countries carry *cagPAI*, whilst many strains from subjects with more benign colonization do not.

1.10.1.2 3' repeat sequence of *cagA*

Sequence analysis of the *cagA* gene reveals that this protein is heterogeneous and varies in size due to the variable repeat sequences of the 3' region of the gene [191,192]. At least four subtypes (A-D) have been described based on their sizes [115,193].

The function of this heterogeneity is not well understood. However it is believed that this variability influences pathogenicity and is also used by the organism to evade the immune system by presenting antigenic diversity or immune dominant non-protective epitopes [59].

1.10.1.3 *vacA*

The *vacA* gene is present in all *H. pylori* strains but only about 50% of the strains have detectable cytotoxin activity [113]. This cytotoxin, which is an important virulence determinant, induces vacuolation of mammalian epithelial cells [194] and also suppresses B and T cell-immunity [195,196].

1.10.1.4 Signal and mid region of *vacA*

The *vacA* gene comprises of two variable parts, namely the signal peptide coding signal region (s) and the mid (m) region [197]. The "s" region also comprises of two alleles *s1* and *s2*, and the *s1* allele has several subtypes

such as *s1a*, *s1b*, *s1c*. The “m” region also has two main alleles namely *m1* and *m2* with the *m1* comprising of the three subtypes *m1a*, *m1b*, *m1c* [21,198]. The presence of various subtypes of signal and mid region leads to a mosaic organization of the *vacA* gene [198]. Certain combinations correlate with the different levels of cytotoxic activity demonstrated by this organization, and correlate with pathogenicity of the organism [198]. The amount of cytotoxin produced is highest with *H. pylori* strains exhibiting the *s1/m1* allele, followed by the *s1/m2* allele. *H. pylori* strains exhibiting the *s2/m1* or *s2/m2* allele show little or no evidence of cytotoxin activity [105].

1.10.1.5 *iceA* gene (induced by contact with epithelium)

The *iceA* gene, which has been identified recently, encodes a homolog of a type II restriction endonuclease [111,199] and has two alleles designated *iceA1* and *iceA2*. The two allelic variants yield either 297bp (*iceA1*) or 229 and 334bp (*iceA2*) products by PCR [197] according to the presence of repeated sequences of 105 nucleotides. These genes have been associated with gastric disease [110,112,199]. In a meta-analysis from articles published through 2011 [200], *iceA1* was significantly associated with peptic ulcer (PU) disease whereas *iceA2* was inversely associated with PU.

Besides *cagA*, *vacA* and *iceA*, the duodenal ulcer-promoting gene A (*dupA*) which encodes a VirB4 ATPase homolog, is also associated with an increased risk of developing duodenal ulcer but a reduced risk of gastric atrophy and gastric cancer [120]. Recent studies have indicated that this could be due to secretion of pro-inflammatory cytokine by mononuclear cells induced by an active *dupA* [201].

1.10.1.6 Extra-chromosomal DNA-plasmid

About 50% of all *H. pylori* carry plasmids [202]; the sizes of these plasmids vary from 1.8-100 kilo bases [203] and are generally cryptic with unknown roles and functions. However, there are some indications that these plasmids might contribute to the genetic diversity of *H. pylori* through multiple recombination events [204].

1.11 Phylogeny

Phylogenetic analyses of both virulent and housekeeping gene sequences from different parts of the world have revealed distinct *H. pylori* gene pools in geographically defined human populations in regions of Asia, Europe, and Africa [81,159,170,205]. Studies have revealed that genetic recombination of *H. pylori* between isolates from different continents is rare [206,207]. This relative segregation of *H. pylori* serves as a useful genetic marker tracking human descent in which an individual spent his/her childhood and also helps track human migration. These analyses have also shown that *H. pylori* followed human migration out of Africa

[159,160] and identified seven *H. pylori* populations which are designated hpAfrica1, hpAfrica2, hpNEAfrica, hpEurope, hpSahul, hpAsia2, hpEastAsia [89,159,208]. Detailed analyses have further subdivided the hpEAsia population into three subpopulations – hspEAsia (common in East Asia, hspmaori (common in Polynesians) and hspAmerind (common in Americas). HpAfrica1 also has two sub-populations – hspWAFrica (common in West Africa) and similar sub-population called hspSAfrica which is common in South Africa.

The African strains (hspWAFrica (West), hspSAfrica (South) and hpNEAfrica (North East), form distinct phylogenetic populations but closely related to one another than to those from elsewhere and the very divergent hpAfrica2 found in South Africa. These phylogenetic analyses have led to a proposal that historical patterns of human migration and of racial admixture can be predicted from DNA sequences of *H. pylori* housekeeping genes [159,160,186,209]. For example, in a study in a shanty town, Peru, the population admixture of Amerind/Asian strains found in this area is a reflection of human migration from Asia to the America about 15000 years ago [210]. However, the *H. pylori* strains found in South America were predominantly hpEurope which suggest that the ancestral *H. pylori* strains (hspAmerind) may have been lost or displaced by the much fitter European strain with more competitive advantage [117,210]. Further, in ethnic groups in Malaysia, four different *H. pylori* sub-populations (hpAsia2, hpEasia, hpEurope and hpAfrica1),

were found probably reflecting the migration of Chinese and Indians into Malaysia about 3000 years ago. The presence of hpEurope may have been due to importation by the Indians who have been colonized by Portugal and Britain [207,211].

The clustering of the discrete hspWAfrica, hspSAfrica and hpNEAfrica populations may reflect the expansion of the Bantu people throughout the African continent from an ancestral homeland in or near present day Benin, Cameroon and Nigeria over the last 4000 years. The frequent isolations of hpAfrica1 and hpEurope in the Americas may reflect the transfer of slaves from Africa about three centuries ago and also European colonization. Phylogenetic analysis have shown that in a study in Colombia [117], subjects living in coastal regions who are predominantly “Mulatoes” of African and European descent carried strains that were mainly hpAfrica1 (66%) and hpEurope (34%).

An ability to discern *H. pylori* lineages from distinct human populations should also have medical relevance, because disease outcomes associated with *H. pylori* infection vary geographically [80,212], and some of this variation might stem from genetic differences between *H. pylori* populations [213,214]. A study in Colombia with a high prevalence of *H. pylori* infection, showed a 25-fold increase in gastric cancer risk in one area (high mountain) when compared to coastal regions. Phylogenetic analysis, revealed that all isolates from the gastric cancer high risk area

where all hpEurope whereas those from the coastal region were predominantly hpAfrica1 (66%). Further analyses showed that hpAfrica strains were associated with reduced severity of gastric lesions [117] as compared to those that carried hpEurope strains with more severe pre-malignant histological lesions. Phylogenetic analysis can therefore be a useful tool for clinical management and for enhanced disease surveillance in high risk areas. Further, phylogenetic analysis of several strains from Africa, Europe, Asia and China showed that the phylogenetic origin of *H. pylori* can be a determinant of gastric cancer risk [212].

1.12 Treatment of *H. pylori* infection

When *H. pylori* is detected in patients showing clinical signs of *H. pylori* associated diseases, the normal procedure is to eradicate the bacteria with antibiotics allowing the disease to heal. Over the past two decades, different treatment regimes using a proton pump inhibitor and various antibiotic combinations such as clarithromycin, tetracycline, metronidazole, amoxicillin and fluoroquinolones [215,216] have been recommended and used for the successful treatment of *H. pylori* infection to heal ulcers and prevent relapses. In The Gambia, where *H. pylori* infection is high and *H. pylori* associated disease occur [19], several antibiotics and a proton pump (clarithromycin, amoxicillin, tetracycline, ampicillin, metronidazole and omeprazole) in various combinations depending on availability and affordability are used for the eradication of *H. pylori* (clinical communication). However, all the

recommended regimes can only cure up to about 90% of people [217]. This is of course a great concern particularly to the developing world where the current cost of treatment is often beyond the reach of the ordinary citizen.

In most cases, the diagnosis of *H. pylori* is based on non-culture investigations such as Urea-Breath test, stool antigen or Rapid urease and so routine antibiotic susceptibility is rarely undertaken before antibiotic prescription [218]. This empirical treatment together with other factors including non-compliance of treatment [219], has contributed to treatment and/or eradication failures.

1.12.1 Antibiotic susceptibility

Infection of this bacterium causes a very high rate of morbidity in both developed and developing countries which directly impacts on the burden of health care systems worldwide particularly in resource-poor countries. Therefore local knowledge of antibiotic susceptibility and resistance is important to plan locally appropriate effective treatment.

1.12.2 Metronidazole resistance

In Europe and USA between 12-40% of *H. pylori* strains were reported resistant to metronidazole (Mtz) [216]. The figures are much higher in developing countries especially in Africa where resistance of up to 100% has been reported [169,220,221]. In the strains studied to date, mostly

from industrialized societies, modest level Mtz resistance (e.g. to 8 or 16 µg Mtz/ml) was often associated with inactivation of the gene *rdxA*, which encodes a non-essential oxygen-insensitive NADPH nitroreductase that chemically reduces metronidazole in vitro [222]. Higher level resistance in *rdxA* mutant strains, e.g., to 32 µg Mtz/ml, results from inactivation of *frxA*, a related but generally less strongly transcribed nitroreductase gene. However, according to Jeong et al, the inactivation of *frxA* enhances resistance to metronidazole [222] and that genes conferring resistance to metronidazole without *rdxA* inactivation are rare or non-existent in *H. pylori* populations; yet higher resistance can result from mutations in any of several additional genes that likely also affect intracellular redox potential [222-224]. It is also important that Mtz's hydroxylamine-type derivatives are mutagenic, that exposure to sub-lethal Mtz concentrations [225] both induces and selects for mutations to Mtz resistance.

1.12.3 Clarithromycin resistance

Clarithromycin is a macrolide that is used in combination with other antibiotics to treat gastric diseases associated with *H. pylori* [226]. However, we have seen an increase in the prevalence of *H. pylori* resistance to this macrolide both in developed and developing countries which has been attributed mainly to its increasing use to treat respiratory infections especially in children at least in Europe and USA [216,227].

Between 2-22% *H. pylori* resistance to clarithromycin have been reported in Europe and USA [216]. Studies in Africa have also shown increase in prevalence of clarithromycin and between 4-55% have been reported in various countries [169,228,229].

Most of the resistance to clarithromycin results from two point mutation- A2142G and A2143G- in 23s *rRNA* gene of *H. pylori*, the latter being more predominant [230]. However, there are reports that other mutations in the 23s *rRNA* genes such as T2182C and T2190C were implicated in *H. pylori* resistance to clarithromycin [231].

1.12.4 Amoxicillin and tetracycline resistance

Resistance to amoxicillin and tetracycline is uncommon among *H. pylori* clinical isolates especially in USA and Europe [232,233] accounting for less than 1% and so treatment failures due to resistance to amoxicillin and/or tetracycline is not of great concern. Resistance to amoxicillin is usually mediated by mutations of the genes encoding penicillin-binding proteins [234,235]. For tetracycline, the resistance mechanism is caused by a triple mutation in the 16s *rRNA* gene exhibiting AGA926-928TTC [236,237]. The rarity of tetracycline resistance in clinical isolates may be due to the need for this triple mutation. Thus, the near or universal resistance to amoxicillin and/or tetracycline reported in some studies in Africa [169,221] must therefore be interpreted with caution.

1.12.5 Antibiotic resistance in Africa

In Africa, the pattern of resistance is variable. These differences might be due to the variations in local antibiotic prescription and community availability. For example, in Cameroon [169], resistant rates of 43.9%, 44.7%, 86.4% and 84.4% have been reported for tetracycline, clarithromycin, amoxicillin and metronidazole respectively. In a similar study in Western Nigeria, 100% resistance was reported to amoxicillin, tetracycline and metronidazole [221] whereas in Kenya [220], all strains were resistant to metronidazole but sensitive to clarithromycin, amoxicillin and tetracycline. In Egypt, although there was universal resistance to metronidazole in child isolates [228], resistant against clarithromycin, erythromycin, azithromycin, ciprofloxacin and ampicillin varied between 2-4%.

The steady increase in the rate of resistance to antibiotics used for the treatment of *H. pylori* is a great public health concern especially to the developing countries where the cost of the first line drugs is often beyond the reach of the average citizen. It is therefore important that antibiotic resistant levels are continuously monitored to help clinicians effectively manage patients and effectively deal with the ever increasing high rate of treatment failures. It is also important that cheaper drugs such as erythromycin are tested as a suitable alternative to the already high cost of *H. pylori* eradication antibiotics.

1.13 Prevention of *H. pylori* infection

The ability of *H. pylori* to survive, evolve and re-emerge for almost a decade after treatment [238] has also contributed to treatment failures. The increase in treatment failures has heightened the need for effective prevention strategies [239]. It has been shown that transmission occurs mainly in childhood [186], seems to run in families [89] and is more common in crowded and unclean conditions [240] which suggests that *H. pylori* infection may be contagious. As there are currently very little or no defined guidelines to prevent transmission, it's always important to observe simple and effected hygiene such as washing of hands, drinking clean and safe water and eating food that is properly cooked.

1.13.1 Vaccines

Prevention of *H. pylori* by improving personal hygiene and sanitation in resource poor countries will continue to be a challenge as clean drinking water and proper drainage systems are almost non-existent in some communities. Therefore, to achieve a global eradication of *H. pylori*, a safe, effective and cost-effective vaccine seems to be the ultimate approach. There have been extensive researches in the past two decades to find a suitable candidate vaccine with varying degree of success [241-243]. However, some studies have shown promising results [244], suggesting that the stimulation of a Th2 response may be a key to immunity against *H. pylori* infection [139]. Vaccination with a wide range of antigens, adjuvants, and delivery routes produced statistically

significant reductions in *H. pylori* colonization levels in mice [245]. These have not been tested in humans and it is unclear whether similar reductions in bacterial load can be achieved. In addition, a multiepitope DNA vaccine containing eight proteins namely FlaA, UreaA, CagA, VacA, HpaA, KatA, NapA and TsaA [246] and another containing the chaperonin GroEL; the external membrane protein HomB; and the highly virulent marker VacA protein are being evaluated [247]. DNA vaccines are live attenuated vaccines and provide important advantages as they mimic the effects of natural infection in their ability to endogenously express foreign protein and also induce humoral as well as cellular immune responses [247].

However, the development of a suitable vaccine has been affected by the lack of understanding of the molecular basis of protection that can lead to complete eradication of the bacterium [248], relative lack of good understanding of the complex gastric immune response and the heterogeneity of *H. pylori*. In addition to this, finding the best efficacious method of vaccine delivery has also been a challenge. Although several methods such as oral, nasal, rectal and systemic immunization have been shown to confer protection in mice [249], the availability of a suitable adjuvant and generation of adequate immunologic response in the stomach continue to pose problems. Recent advances in *H. pylori* genomics and an increase understanding of the gastric immune response provide opportunity to accelerate progress in *H. pylori* vaccine

development [250]. There is evidence of passive immunity against *H. pylori*. A study in The Gambia has shown that specific antibodies in milk directed against known bacterial colonization factors, such as urease, appear to protect against *H. pylori* colonization in infants [251].

The increase in antibiotic resistance, poor compliance, inaccessibility and unavailability of effective antibiotics especially in resource poor countries makes the need for the development of a suitable vaccine more urgent.

Chapter 2 Hypotheses, aims and objectives

This study of Gambian *H. pylori* strains was motivated by considering that *H. pylori* is a genetically diverse bacterial species, with different genotypes predominating in different well separated geographic regions. However, most of the detailed studies and analyses of *H. pylori* have been conducted in strains from industrialized countries. There have been far fewer robust studies of *H. pylori* strains from Africa, especially those from West Africa. It is therefore important that a detailed genetic study of the *H. pylori* isolates in The Gambia is carried out in order to explore *H. pylori* strains from a relatively unstudied population, to understand the development of *H. pylori* associated diseases in The Gambia, West Africa and to compare them with strains from other geographically defined populations to help address important questions in this area.

2.1 Hypotheses

1. Putative virulence factors such as *cagA* and *vacA* influence disease outcome in The Gambia.
2. Mixed colonization with different genotypes influences the development of gastroduodenal disease
3. Children and adults in the Gambia are colonized by different strains of *H. pylori*, which therefore circulate amongst different age groups
4. Gambian isolates of *H. pylori* form a distinct phylogenetic family within the grouping of African derived strains.

2.2 Aims and objectives

The primary aim of this study is to characterise and evaluate the putative virulent *H. pylori* genotypes in The Gambia in relation to clinical outcome.

2.3 Objectives

1. Determine the prevalence of *H. pylori* genotypes in The Gambia in relation to disease expression.
2. Determine the genotypes of child strains and compare with adult strains.
3. Investigate the phylogenetic relationship between strains in The Gambia, and those from other geographically defined populations.
4. Determine the genetic relatedness and phylogenetic of isolates in a single stomach.
5. Determine the prevalence of antibiotic susceptibility and resistance to amoxicillin, clarithromycin, erythromycin, metronidazole and tetracycline.
6. Investigate the usefulness of PCR in the diagnosis of *H. pylori* in The Gambia.

Chapter 3.0 Materials and methods

3.1 Clinical settings at the MRC endoscopy unit

The MRC has an endoscopy unit which is the only facility that offers upper gastric endoscopy in The Gambia and serves as the national endoscopy referral centre. Patients were referred to the unit from throughout The Gambia, principally directly from the MRC Out-patient Clinic or the Royal Victoria Teaching Hospital (RVTH) in Banjul and other privately owned clinics. The principle presentation was dyspepsia, but dysphagia and gastrointestinal haemorrhage were also investigated by upper gastric endoscopy.

3.2 Study population

The study population comprised subjects who attended the MRC Out-patient department (OPD) and were sent to the endoscopy unit as part of the routine clinical investigations or referred from the main hospital in Banjul (RVTH) or from other private clinics. Adult subjects who consented to join the study were recruited from among these out patients undergoing diagnostic upper gastric endoscopy primarily for symptoms of dyspepsia. The small group of young children comprised patients with significant growth faltering associated with enteropathy undergoing endoscopy in order to obtain small bowel biopsies as part of an investigation into the underlying nature of their condition.

3.3 Subject inclusion and exclusion criteria

3.3.1 Inclusion criteria

All patients referred for diagnostic endoscopy to the MRC Unit were eligible for inclusion.

3.3.2 Exclusion criteria

Any contraindication to endoscopy and biopsy, such as liver dysfunction, history of bleeding, history of other serious organ disease (for example, kidney, heart, lung), bleeding time prolonged to over 10 minutes, or unexpected findings such as oesophageal or gastric stricture that limits endoscopic examination excluded subjects from the study, as did the use of antibiotics during the previous two weeks. Patients with severe oesophago-gastroduodenal disease, including those with gastro-oesophageal varices and a small number with advanced gastric cancer were also excluded from the study. In addition, if the endoscopist felt that prolonging the procedure by 2 or 3 minutes in order to take the research biopsies would not be advisable for any reason, the subject was also excluded from the study.

3.4 Good Clinical Practice (GCP)

This study was conducted according to GCP and conformed to the International Conference on Harmonisation Good Clinical Practice E6 (R1) (ICH-GCP) and the following specific regulatory requirements:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR 46 and 21 CFR including Parts 50 and 56 concerning informed consent and Institutional Review Board [IRB] regulations) which regulates the protection of human subjects in research supported or conducted by the Department of Health and Human Services.
- Completion of Human Subjects Protection Training

The endoscopy team of MRC comprised of three Medical doctors assisted by two SRN nurses and four SEN nurses. As part of GCP requirements, the principal investigators of this study completed a "Human Participant Protection Education for Research Team" on-line course sponsored by the NIH. The whole endoscopy team also completed a Family Health International Certificate course on Research Ethics Training curriculum and also on the Protection of Human subjects in Clinical Research. In addition, the lead endoscopist and I, as study coordinator, completed a computer based Clinical Research Training course. Furthermore, all nurses and laboratory technicians involved in this study completed a locally organized introductory training on Good Clinical Practice (GCP). Standard endoscopy procedures (SOP-CLS-001) were followed; using clean endoscopes sterilized with Cidex (Johnson and Johnson Co) and rinsed with clean water between cases, according to standard care at MRC Unit, The Gambia.

3.5 Study monitoring

Following ethical review, NIH monitors undertook annual audits to ascertain record keeping, data management, clinical and laboratory processes to GCP and GCLP standards. The local Clinical Trial Support Team also undertook annual audits to review progress and study conduct. In addition, an onsite Independent Safety Monitor [ISM (who was not involved in the trial)] was selected by the Principal Investigator (PI) and approved by DMID based on relevant study related experience and lack of conflict of interest to independently and thoroughly review all serious adverse events and to communicate in writing his/her findings, concerns and recommendations to DMID and the study principal investigator.

3.6 Study approvals

This study was carried out under the supervision and approval of The Gambia Government/MRC joint ethics committee (appendix A-ethics letter) and Division of Microbiology Infectious Diseases (DMID) International Review Board of USA with a protocol number of DMID 06-0053 and an MRC Unit, The Gambia IRB registration number: IRB00003943 and a Federal Wide Assurance number: FWA 00006873. FWA is the number indicating that all documents relevant to human subject protection has been submitted to the office of Human Research Protection.

3.7 Subject enrollment and consent procedure

3.7.1 Adult subjects

Adult subjects who were referred to the endoscopy unit for upper gastrointestinal endoscopy had the study described to them in English or a local language that they can understand. The study information sheet (appendix A- adult information sheet) was also given to them to read or interpreted to them in a local language that they can understand and they were asked if they would consent to additional biopsies (approximately 10 to 14mg) being collected from the gastric antrum, corpus, and cardia for research purposes during the procedure (appendix A- adult consent form). A signed consent was obtained at this stage. The study subjects were not compensated for study participation. Those who declined (259) went on to have their procedure as planned but without the collection of additional biopsies.

3.7.2 Young Children

Young children who were referred to the endoscopy unit because of persistent diarrhoea, malnutrition and suspected enteropathy had the procedure described to the mothers or guardian at length before hand, which included showing the mother or guardian the endoscope and biopsy forceps prior to the child being prepared for the procedure. This study was then explained to the mothers/guardians, who were asked if they would consent to additional biopsies (approximately 5 to 7mg) being collected

for the purposes of research. No compensation for participation was offered to the parents or guardians who consented for their children to join the study. A signed consent was obtained at this stage (appendix A-child consent form). The mothers/guardians were encouraged to be present during the procedure if they wished; several stayed with their children, and a "running commentary" was provided in their language by one of the team who speaks a local language that the mother or guardian understands and to confirm at the appropriate juncture that the parent/guardian was still happy for research biopsies to be taken once they had a complete understanding of the processes involved. If parents consented, and the endoscopist considered it appropriate, additional biopsies were collected from the gastric antrum before the endoscope was withdrawn. Those children whose guardians or parents declined (12) went on to have their procedure as planned but without the collection of additional biopsies.

3.7.3 Consent supervision

The study information and consenting processes were supervised by the endoscopist who was present through out to answer any question from the study subjects. There was always an independent witness to the consenting process who was required to sign the consent form.

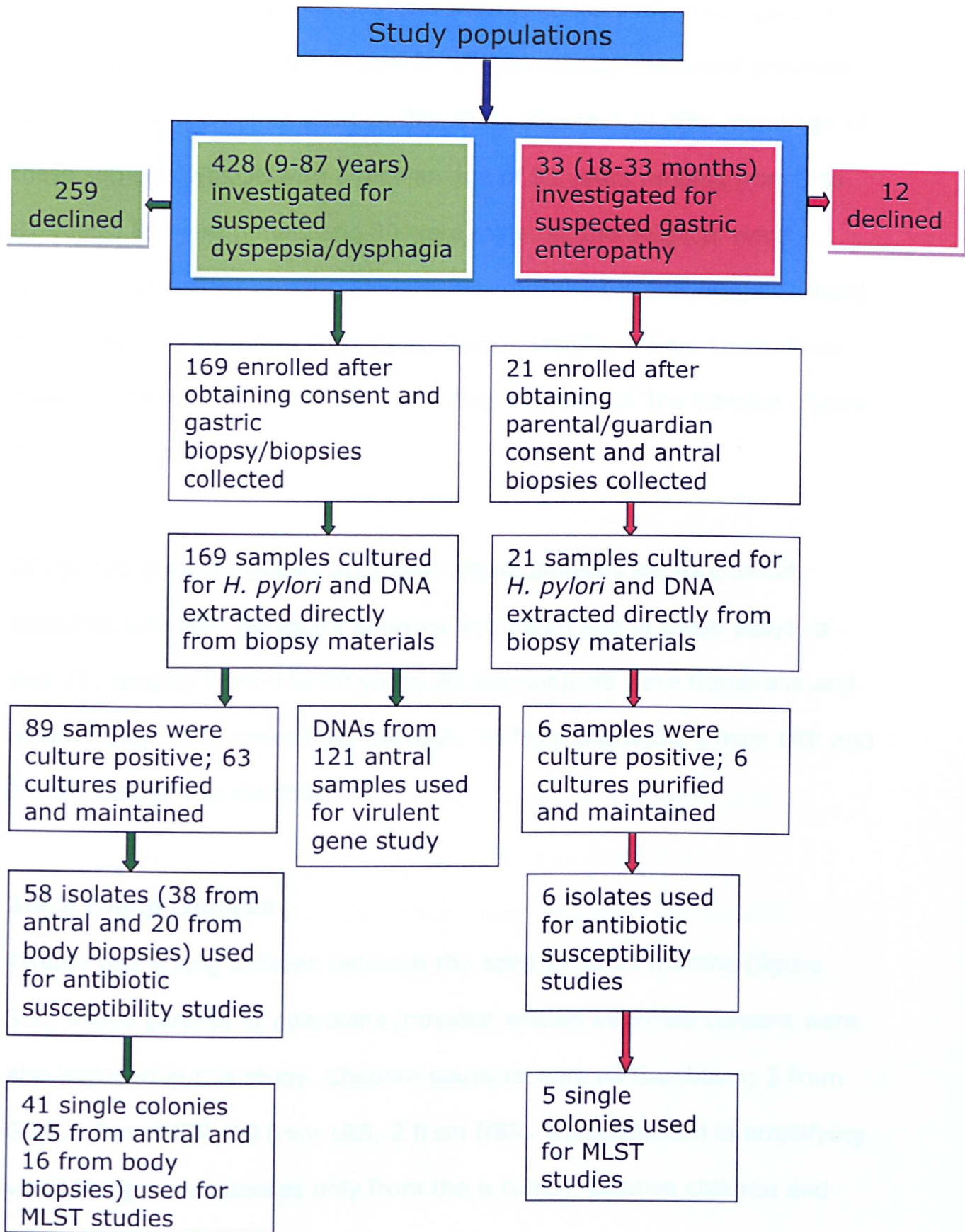
3.7.4 Data management and security

Each subject was assigned a study number, and this was used to identify all biopsy samples, *H. pylori* isolates and DNA extracts obtained from individual subjects. All records are kept at the MRC Unit in Fajara, The Gambia and data processes and record keeping were annually monitored by NIH agents. *H. pylori* isolates and DNA extracts transported to Washington University in St. Louis, Missouri, USA for antibiotic susceptibility tests were identified by a study number alone. Only senior MRC laboratory staff and the principal investigator were able to match isolates with individual clinical records at the MRC Unit, The Gambia.

3.8 Patients referred for endoscopy

A total of 428 patients between 2003 to 2008 were investigated by upper gastric endoscopy for routine clinical diagnoses in the MRC endoscopy unit. These were patients either seen at the MRC-OPD and referred to the MRC endoscopy unit as part of clinical investigations for dyspepsia or patients referred from other hospitals or clinics from around the country. In addition, there were 33 young children between the ages 18-33 months who were referred to the unit for endoscopy because of enteropathy and malnutrition (figure 3.1).

Figure 3.1 Flow chart of study samples



3.8.1 Study participants

One hundred and sixty nine subjects who were routinely investigated by upper gastrointestinal endoscopy for clinical management and provided written consent were involved in this study (figure 3.1). The mean age of these subjects was 38 with a median age of 33 years ranging from 9 to 87 years; 89 were female and 80 were male. All the subjects were Gambians and most of them (111) came from the Greater Banjul (urban) Area (GBA), 42 from the West Coast Region (WCR), 9 from Lower River Region (LRR) and 7 from North Bank Region (NBR) of The Gambia (figure 1.1).

Of the 121 patients (figure 3.1) from whose biopsies we successfully amplified virulence genes for analysis, the mean age of these subjects was 35, ranging from 9 to 80 years. All the subjects were Gambians and most of them (75) came from the GBA, 38 from the WCR, 5 from LRR and 3 from NBR of The Gambia.

3.8.2 Young children

Twenty one young children between the ages 18 to 31 months (figure 3.1) whose parents or guardians provided written informed consent were also included in this study. Children subjects were all Gambians; 3 from GBA, 6 from WCR, 10 from LRR, 2 from NBR. We succeeded in amplifying virulence gene sequences only from the 6 culture positive children and these were the ones included in the final analyses.

3.8.3 Specimens

3.8.3.1 Gastric biopsies

Gastric biopsies were collected from subjects who consented to join the study and underwent diagnostic endoscopy because of dyspeptic symptoms or suspected enteropathy as part of routine investigation into the underlying nature of their condition. The gastric biopsies collected were immediately stored in ice and transported to the laboratory for culture and/or DNA extraction.

3.9 Good Clinical Laboratory Practice (GCLP)

The gastric biopsies collected were stored in Brain Heart Infusion (BHI) broth containing 20% glycerol immediately after collection and transported on ice from the site of collection to the laboratory for processing or stored at -70°C until used. Pipettes used in all experiments were verified and calibrated according to standard protocol. All media used for the culture of *H. pylori* were sterilized using an autoclave and poured on Petri-dishes in a lamina flow cabinet to minimise contamination. Sterile disposable loops were used for culturing and spreading of biopsy samples and cultures. Despite the use of antibiotics and antifungal in culture media, the cultures were sometimes overgrown with contaminants. To minimize the level of contamination, a ClassII safety cabinet was used during the laboratory processing of samples. The gas chamber was disinfected with 2% Virkon and carbon catalysts

sterilized by autoclaving each week. All biohazard materials were disposed off according to the local Health and Safety regulations (SOP-H&S-001).

3.10 Detection of *H. pylori*

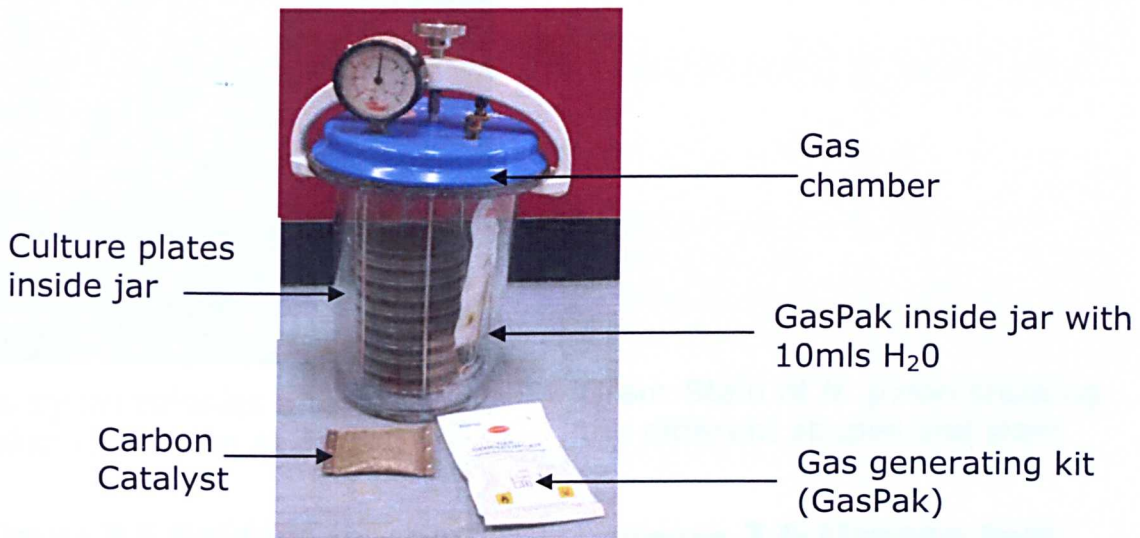
In this study, the detection of *H. pylori* was by culturing the bacterium on solid selective media and isolating suspect colonies and identifying them using standard microbiological techniques and also by PCR using *H. pylori* specific primer (*Hp16s rRNA*).

3.10.1 Bacteriology

3.10.1.1 Culture of *H. pylori*

Endoscopic biopsies were spread over the surface of a Columbia-blood agar (Unipath Ltd, Basingstoke, UK) media plate supplemented with 10% horse blood (TCS Biosciences, UK) and 2% vitox (Unipath Ltd, Basingstoke, UK). To minimize contamination, trimethoprim (5µg/ml), vancomycin (6µg/ml), polymixin B (10µg/ml), bacitracin (200µg/ml), nalidixic acid (10µg/ml) and amphotericin B (8µg/ml) were added to the Columbia-blood agar and inoculated plates were immediately put in a gas chamber under micro-aerobic atmosphere composed of 86% Nitrogen, 4% Oxygen, 5% Carbon dioxide and 5% Hydrogen. This atmosphere was generated by the use of Campylobacter Gas generating kit (Oxoid, UK) in the presence of a carbon catalyst and the plates were then incubated at 37°C for 4-7 days.

Figure 3.2 Gas generating system using GasPaks.



3.10.1.2 Isolation and identification

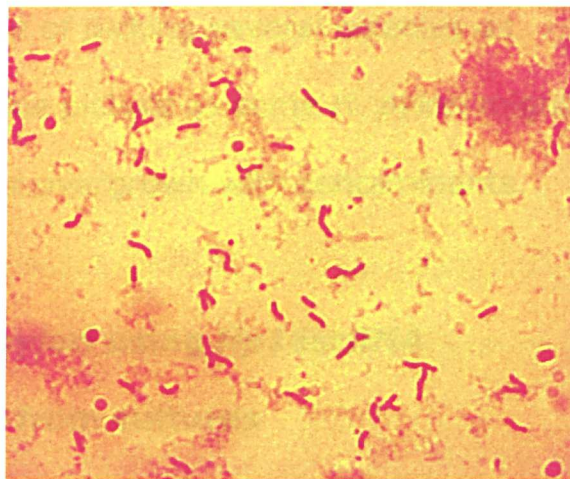
Identification of *H. pylori* was by standard methods including colony morphology, Gram stain appearance, urease, oxidase and catalase activities. On 10% Columbia-Blood agar, *H. pylori* colonies were grey, convex and translucent colonies of 1-2mm diameter (figure 3.3). They were urease, oxidase and catalase positive and Gram-negative bacilli showing different shapes and sizes (figures 3.3-3.7).

Figure 3.3 Bacterial culture of *H. pylori*



H. pylori colonies after 4 days incubation at 37°C

Figure 3.4 Gram stain of *H. pylori*



Gram-Stain of *H. pylori* showing the different shapes and sizes

Figure 3.5 Oxidase reaction

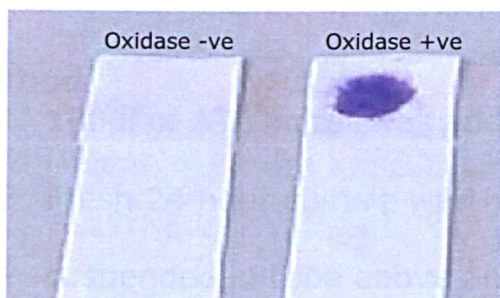


Figure 3.6 Urease test

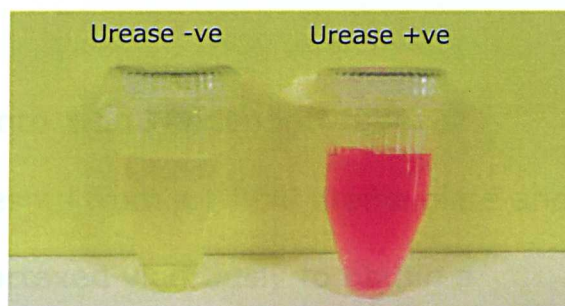
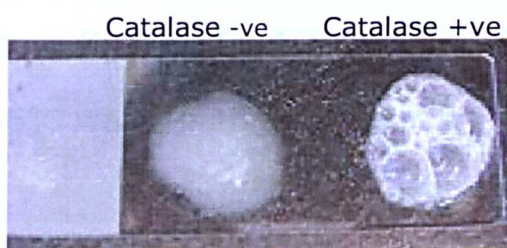


Figure 3.7 catalase reaction



During preliminary studies and also during the study, this technique of culturing *H. pylori* became challenging with cultures overgrown with fungal as well as other bacterial contaminants despite the use of a selective media. Thus, it was necessary to find an optimal method such as polymerase chain reaction (PCR) to detect *H. pylori* in this challenging environment and also to accurately evaluate the prevalence of mixed *H.*

pylori infections in a host. The method of picking several colonies as pooled cultures may not reflect the true picture as some *H. pylori* might be un-culturable, die during culture or overgrown with contaminants.

3.10.2 Molecular detection of *H. pylori* using *Hp16srRNA*

3.10.2.1 Genomic DNA extraction from cultures

Genomic DNA was prepared by harvesting a 24 hour confluent growth of *H. pylori* culture and extracted using a commercial kit (Qiagen DNA Mini Kit, UK) as per manufacturers' guidelines described below:

Method

1. 180µl of ATL buffer was added into 1.5ml eppendorf tube.
2. Fresh 24 hour culture was harvested from a whole media plate and suspended in tube above and vortexed vigorously to obtain a homogeneous suspension
3. 10µl of proteinase K was added, mixed for 15 seconds and incubated at 56°C water bath for 1 hour, mixing at 15 minutes intervals
4. 200µl of AL buffer was added, mixed for 15 seconds and incubated at 70°C for 10 minutes
5. 200µl of 96-100% ethanol was added, mixed for 15 seconds, transferred fluid into a spin column provided in Kit and centrifuged at 8000rpm for 1 minute and flow-through discarded

6. The spin column was put into a new collection column and 500µl of AW1 buffer added, mixed for 15 seconds, centrifuged at 8000rpm for 1 minute and flow-through discarded
7. The spin column was put into another collection column, 500µl of AW2 buffer added, centrifuged at 14000rpm for 3 minutes and flow-through discarded.
8. The column was put in a clean 1.5 eppendorf tube, 200µl of AE buffer (elution buffer) added and incubated at room temperature for 5 minutes to increase DNA yield.
9. The tubes were centrifuged at 7000rpm for 1 minute and column discarded.
10. Fluid in eppendorf about 200µl was the extracted total DNA
11. The DNA was stored at -20°C until used.

3.10.2.2 Genomic DNA extraction directly from biopsies

Total genomic DNA was extracted from the biopsy samples by using a combination of the QIAamp DNA isolation kit (Qiagen) and a bead-beater method as described below:-

1. The biopsy samples were removed from the transport media and transferred in a sterile 1.5ml eppendorf tube and 180µl of ATL buffer added into the 1.5ml eppendorf tube.
2. 20µl of proteinase K was added mixed and vortexed vigorously to lyse the biopsies.
3. The suspension in the tubes were incubated at 56°C water bath for 1 hour, mixing at 15 minutes intervals.

4. Glass beads of different diameters (0.1mm, 0.5mm and 1mm, Sigma, USA) were added to maximize contact and optimize homogenisation.
5. Samples were then homogenized in a FastPrep FP120 bead beater (Bio101, Savant Instruments) for 30 sec at 4 m/s and incubated for an additional hour at 56°C mixing at 15 minutes intervals.
6. 200µl of AL buffer was added, mixed for 15 seconds and incubated at 70°C for 30 minutes.
7. 200µl of 96-100% ethanol was added, mixed for 15 seconds, fluid transferred into a spin column provided in Kit and centrifuged at 8000rpm for 1 minute and flow-through discarded.
8. The spin column was put into a new collection column and 500µl of AW1 buffer added, mixed for 15 seconds, centrifuged at 8000rpm for 1 minute and flow-through discarded.
9. The spin column was put into another collection column, 500µl of AW2 buffer added, centrifuged at 14000rpm for 3 minutes and flow-through discarded.
10. The column was put in a clean 1.5 eppendorf tube, 200µl of AE buffer (elution buffer) added and incubated at room temperature for 5 minutes to increase DNA yield.
11. The tubes were centrifuged at 7000rpm for 1 minute and column discarded.
12. Fluid in eppendorf about 200µl was the extracted total DNA.
13. The DNA was stored at -20°C until used.

3.10.2.3 Molecular detection of *H. pylori* 16srRNA gene

PCR was performed to detect *H. pylori* 16srRNA gene as previously described [252] using the primers listed in table 3.1 and the following cycling conditions: 30 cycles of 30 seconds at 95°C, 30 seconds at 55 or 60°C, and 30 seconds at 72°C. The amplified genes were detected by electrophoresis in a 1.5% gel with ethidium bromide (500ng/ml) and bands visualized using Gel Doc 2000 (Bio-Rad laboratories, Milan, Italy). The presence of *H. pylori* was inferred when a product of the expected size (109bp) was obtained using appropriate primers (table 3.1). A 100bp DNA ladder (Biolabs, UK) was used as a size marker in all gels. To determine the specificity of the *Hp16srRNA* primers, DNA was extracted from faecal material, *E. coli*, *Staphylococcus aureus*, *Salmonella* and *Streptocococcus pneumoniae* and amplified with Hp1 and Hp2 primers. These DNAs all tested negative.

3.11 Antibiotic susceptibility to *H. pylori*

3.11.1 Minimum Inhibitory Concentration (MIC) by agar dilution

Methods:

1. *H. pylori* strains were grown on selective media as described before (section 3.10.1.1).
2. The test media were prepared with the following antibiotic concentrations:
 - amoxicillin 2µg/ml
 - clarithromycin 2µg/ml

-erythromycin 2µg/ml

-metronidazole 8µg/ml, or 16, 32, 64, 128, 264µg/ml

-tetracycline 2µg/ml

-ordinary plate without these antibiotics but always contains the three antibiotics –trimethoprim, vancomycin and amphotericin-[253] that do not act against *H. pylori* to suppress possible contaminating bacteria and moulds.

3. Plates were pre-warmed to room temperature

-NB: tetracycline is photosensitive, therefore plates were wrapped up in foil and stored in a brown box prior to use.

4. Fresh 24 hour *H. pylori* cultures were harvested for this task.

5. 500µl of sterile phosphate buffered saline pH7.2 (PBS, GIBCO, invitrogen, USA) was dispensed into 1.5ml sterile eppendorf tubes

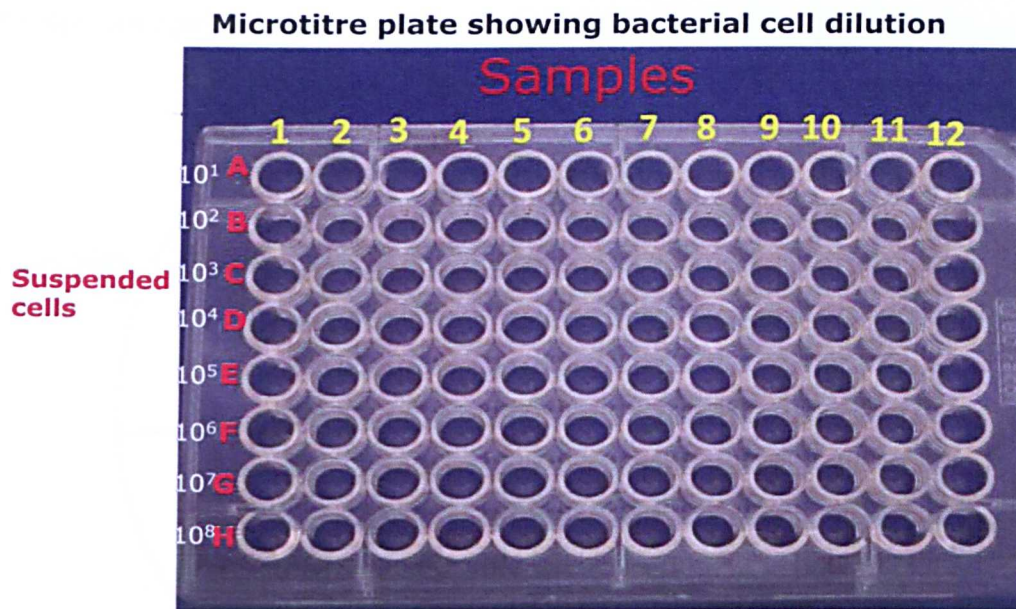
6. *H. pylori* cells were suspended to 10⁵ McFarland standard and vortexed to obtain a homogeneous suspension.

7. 90µl of sterile PBS was dispensed into micro-titre wells A to H (figure 3.8).

8. Into well A, 10µl of suspended cells was added (step 6) and mixed thoroughly.

9. Series of 10 fold dilutions A to H were made by transferring 10µl of dilution from well A to B, then B to C, etc to the last well, changing pipette tips in between.

Figure 3.8 Micro-titre plate showing dilution of cells



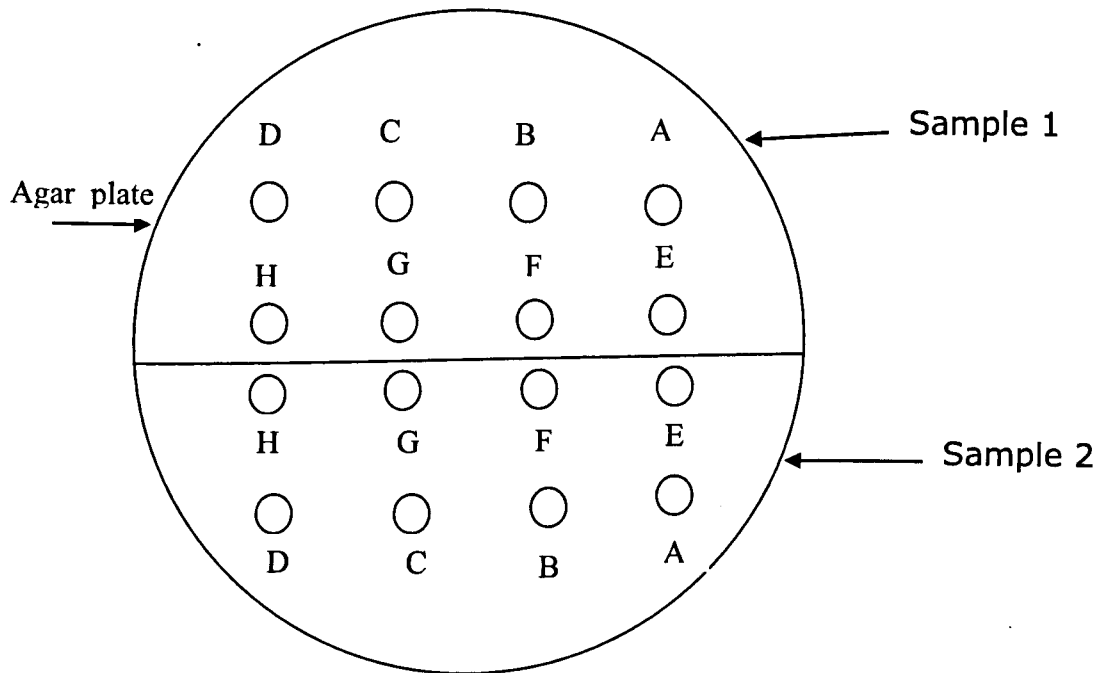
10. From each dilution, 10 μ l was spotted on appropriate plate starting with the lowest dilution (well H).

NB: an ordinary plate with each test strain was included for comparison, to estimate number of bacteria in the original suspension, and thereby the frequency of cells in the population resistant to the tested antibiotics.

11. Two cultures were tested per plate as indicated in figure 3.9:

concentrations of antibiotics that caused at least a 10-fold decrease in the efficiency of colony formation by individual cells (efficiency of plating, or EOP).

Figure 3.9 Culture plate showing spot inoculums of cell suspension



Key
 ○ = 10 μ l spots
 A-H = 10 fold dilutions 10^1 to 10^8

12. Inoculums were allowed to air dry and plates incubated in a micro-aerobic atmosphere at 37 $^{\circ}$ C for 5 days.
13. Colony count was recorded.

Results = A strain was considered to be susceptible to concentrations of antibiotics that caused at least a 10-fold decrease in the efficiency of colony formation by individual cells (efficiency of plating, or EOP).

3.11.2 *rdxA* (nitroreductase) gene transformation analysis

Resistance of *H. pylori* from other (non-West African) populations to metronidazole is mainly due to inactivation of *rdxA* or both *rdxA* and *frxA* nitroreductase genes, depending on the strain (and whether *frxA* is highly expressed or not). Metronidazole sensitive (Mtz^S) strains generally have functional alleles of these two genes. Given that Mtz^S *H. pylori* strains are relatively uncommon in The Gambia; we elected to test if those that had remained Mtz^S were very different from metronidazole resistant Mtz^R strains in terms of ease of development of resistance or if they had simply not been sufficiently exposed to the antibiotic to have selected for emergence of resistance.

Method

1. Mtz^S *H. pylori* strains were sub-cultured on antibiotic free agar plate.
2. The cells were harvested and sub-cultured again for 24hours to obtain an exponential growth.
3. 24 hour cultures were transferred in a fresh antibiotic free agar plate.
4. 5µl of genomic DNA (ordinary Qiagen prep) from strain that contains a chloramphenicol resistance (CamR) cassette inserted at the *rdxA* locus (*rdxA*-null mutant) was added to the freshly cultured bacterial cells and incubated over night at 37°C.
5. Some bacterial cells would have taken the DNA.

6. The cells were then harvested and subcultured on an agar plate with chloramphenicol to select out CamR strains.
7. The cells of CamR colonies were scraped with sterile toothpick and streaked on agar plate with 8µg/ml of Mtz, and then on control plate with chloramphenicol 15µg/ml.
8. Growth on the Mtz plate indicated that for these strains, inactivation of only *rdxA* gene resulted in resistance.
9. No growth on Mtz plate, inactivation of a second gene (*frxA*) may be necessary for these strains to become resistant. This can be tested with genomic DNA from a strain that contained a kanamycin resistance gene cassette inserted next to a functional *rdxA* gene. We expected that a fraction of Kan^R transformants would acquire the donor strain's *rdxA*⁺ and become sensitive whilst others that did not acquire the functional *rdxA* will remain resistant.

3.11.3 16S rRNA and *rdxA* DNA sequencing

To detect changes in 16S rRNA sequences associated with Tet resistance, 16S rRNA genes were amplified by PCR with primers 16S-F (5'-CGGTTACCTTGTTACGACTTCAC-3') and 16S-R (5'-TATGGAGAGTTTGATCCTGGCTC-3') and the amplified 16S rDNAs were sequenced as previously described [236].

For detection of mutations associated with Mtz resistance, the *rdxA* gene of 51 (33 Mtz^R and 18 Mtz^S) strains was amplified by PCR with the

following primers (rdxAF 5'GTTTCGTTAGGGATTTTATTGTATGCTA-3' and rdxAR 5'CACCCCTAAAAGAGCGATTAAAACCATT-3'), PCR products were sequenced, and the sequences were edited, aligned and analysed using DNASTar programme (Lasergene, USA, Version 7) and Clustalw2 programme.

3.12 PCR amplification for virulent genes (*cagA*, *vacA*, *iceA*)

The extracted DNAs were tested by PCR for the presence of *H. pylori* using *H. pylori* specific *16srRNA* primers. The *H. pylori* positive samples were further tested for the *cagA* oncogene, signal (*s1* and *s2* alleles) and middle regions (*m1* and *m2* alleles) of the *vacA* toxin gene, *iceA1* and *iceA2* genes as previously described [21]. Typically 25µl containing 1U of *Taq* polymerase (Bioline, UK), 10pmol of each primer per reaction, 0.25mM (each) of deoxynucleoside triphosphate, and 2mM MgCl₂ in standard PCR buffer and 1µl of DNA were carried out in a PTC 200 DNA Engine cycler, under the following general cycling conditions: 30 cycles of 94°C for 1 minute, 55°C or 60°C for 1 minute and 72°C for 1 minute. Positive DNA from previously determined genotypes and template free controls that contained only the reaction mixture without DNA were included in each batch as positive and negative controls respectively. The presence of a particular gene or allele was inferred when a product of the expected size (table 3.1) was obtained using appropriate primers. A 100bp DNA ladder (Biolabs, UK) was used as a size marker in all gels.

Table 3.1 Primers used for virulent gene and RAPD

Region	Primer	Nucleotide sequence	bp	reference
<i>cagA</i>	cagA-F cagA-R	gat aac agg caa gct ttt gag g ctg caa aag att gtt tgg cag a	349	[21]
<i>cag empty site</i>	Luni-1 R5280	aca ttt tgg cta aat aaa cgc tg ggt tgc acg cat ttt ccc tta atc	535	[21]
<i>vacA s1 & vacA s2</i>	Va1-F Va1-R	atg gaa ata caa caa aça cac ctg ctt gaa tgc gcc aaa c	s1 259 s2 289	[21]
<i>vacA m1a</i>	Va3-F Va3-R	ggt caa aat gcg gtc atg g cca ttg gta cct gta gaa ac	290	[21]
<i>vacA m2</i>	Va4-F Va4-R	gga gcc cca gga aac att g cat aac tag cgc ctt gca c	352	[21]
<i>iceA1</i>	1048-F 1345R	gct tgt aac gat aag aaa cgc cag at gga atg agc ttg tat tta gag ccg at	297	[21]
<i>iceA2</i>	ICEA2-F ICEA2-R	gtt ggg tat atc aca att tat ttr ccc tat ttt cta gta ggt	229 or 334	[21]
<i>H. pylori 16sRNA</i>	Hp1 Hp2	ctg gag aga cta agc cct cc att act gac gct gat tgt gc	109	[252]
RAPD 1254	1254	ccgcagccaa		[254]
RAPD 1283	1283	gcgatcccca		[254]

3.13 PCR amplification for Randomly Amplified Polymorphic DNA (RAPD)

PCR for DNA finger printing (RAPD) was carried out with two arbitrary primers as previously described [254]. 25µl containing 1U of *Taq* polymerase (Bioline, UK), 10pmol of one primer per reaction, 0.25mM (each) of deoxynucleoside triphosphate, and 4mM MgCl₂ in standard PCR buffer and 1µl of DNA were carried out in a PTC 200 DNA Engine cycler, under the following general cycling conditions: 45 cycles of 94°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes. Primer 1283 (5'-GCGATCCCCA- 3') was used to type the selected isolates to confirm the results of the first primer 1254 (5'-CCGCAGCCAA- 3').

3.14 Gel electrophoresis for virulence genes and RAPD

8µl of the amplified genes or fragments were detected by electrophoresis in a 1.5% agarose gel with ethidium bromide (500ng/ml) after the addition of 2µl of gel loading solution (Sigma, USA) as described below:

1. 1.5% agarose (Sigma, USA. Cat.no. A-9414) gel was prepared by weighing out 1.5g of pure agarose and suspending in 100ml of 1xTBE buffer in a conical flask, dissolved by boiling in a microwave for 3 minutes and allowed to cool to 56°C in a water bath.
2. 100µl of ethidium bromide solution was added to a concentration of 500ng/ml; the gel poured into the carrier on a level surface with the plastic combs already inserted and allowed the gel to set and the combs removed.
3. Ensuring the power pack was switched off; the gel was transferred to the electrophoresis tank and covered with 1xTBE buffer to about 0.5cm above the gel.
4. 2µl of the running dye (loading buffer) was transferred into a u-shaped micro-titre plate. One well was required for each reaction.
5. 8µl of PCR product was removed from the PCR tube, mixed with the running dye by reflux action in the pipette tip and loaded into the gel by introducing the tip under the surface of the buffer and into the well. (The density of the glycerol in the dye made the product sink into the well).
6. The gel tank was covered with the lid and checking that the polarity was correct (DNA moves towards the anode), the voltage was set to 100V and

the process ran for 1 hour. For **RAPD**, the voltage was set at 75V and the process was allowed to run for 4 hours.

7. When the electrophoresis was complete, the gel was transferred to a sandwich box and carefully moved onto the UV box. The gel was positioned and the camera focused.
8. After putting on UV protective glasses, UV light was turned, the gel examined and a photograph taken using Gel Doc 2000 (Bio-Rad laboratories, Milan, Italy) and analyzed.

100bp and 1Kb DNA ladders (Biolabs, UK) were used as size markers in all RAPD gels.

3.15 Multi-Locus sequence Typing (MLST) using seven House-keeping genes

3.15.1 MLST Gene Amplification

MLST was carried out by PCR using seven house-keeping genes loci (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI* and *yphC*) in order to characterize the *H. pylori* strains using sequence analysis. The extracted DNA were amplified with both the forward and reverse primers (amplification primers are listed at <http://pubmlst.org/helicobacter>) of each house keeping gene under the following conditions: 30 cycles of 94°C for 40 seconds, 50°C for 40 seconds and 72°C for 1 minute. The primers used for all PCR amplifications and sequence reactions for MLST are listed in table 3.2.

Table 3.2 MLST Primers used for both amplification and sequencing

Name	Gene	Primer sequence	Reference
<i>atpA</i>	<i>atpA</i> forward	ggactagcgttaaacgcacg	http://pubmlst.org/helicobacter
	<i>atpA</i> reverse	cttgaaaccgacaagcccac	http://pubmlst.org/helicobacter
<i>efp</i>	<i>efp</i> forward	ggcaatttgatgagcgagctc	http://pubmlst.org/helicobacter
	<i>efp</i> reverse	cttcacctttcaagatactc	http://pubmlst.org/helicobacter
<i>mutY</i>	<i>mutY</i> forward	gtggttgtagytggaaactttacac	http://pubmlst.org/helicobacter
	<i>mutY</i> reverse	cttaagcgtgtgtytttctagg	http://pubmlst.org/helicobacter
<i>ppa</i>	<i>ppa</i> forward	ggagattgcaatgaatttaga	http://pubmlst.org/helicobacter
	<i>ppa</i> reverse	gtggggttaaratcgtaaattg	http://pubmlst.org/helicobacter
<i>trpC</i>	<i>trpC</i> forward	tagaatgcaaaaaagcatcgccctc	http://pubmlst.org/helicobacter
	<i>trpC</i> reverse	taagcccgcacactttatttcgcc	http://pubmlst.org/helicobacter
<i>ureI</i>	<i>ureI</i> forward	aggttattcgtaagggtgcg	http://pubmlst.org/helicobacter
	<i>ureI</i> reverse	gtttaaatacccttagattgcc	http://pubmlst.org/helicobacter
<i>yphC</i>	<i>yphC</i> forward	cacgcctatttttgactaaaaac	http://pubmlst.org/helicobacter
	<i>yphC</i> reverse	cattyacccctccaatgatgc	http://pubmlst.org/helicobacter
Tet16S rRNA	<i>Tet16S</i> forward	cggttaccttgttacgactcac	[236]
	<i>Tet16S</i> reverse	tatggagagtttgatcctggctc	[236]

3.15.2 DNA purification for MLST

PCR amplification products for each of the seven house-keeping genes (*atpa*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *yhpC*) were transferred in a sterile 1.5ml eppendorf tube diluted with phosphate buffered saline (PBS) and the contents were processed using a Qiagen purification kit (Qiagen, UK) as described below:-

Method:

1. The whole volume of PCR product was transferred in 1.5ml eppendorf tube and 5X volume of PBS was added and mixed (e.g. 20µl of PCR product + 100µl of PBS)
2. Content was transferred into a spin column, centrifuged at 13000rpm for 1 minute and flow-through discarded.

- 0.75ml of PE buffer was added, centrifuged at 13000rpm for 1 minute and flow-through discarded.
- The spin column was centrifuged again to remove any residual ethanol from the PE buffer.
- The column was transferred into fresh 1.5ml eppendorf tube and DNA eluted with 50µl AE (elution buffer).
- The tubes were incubated at room temperature for 5 minutes to increase DNA yield and centrifuged at 6500rpm for 1 minute.
- The resulting purified DNA was stored at -20°C until used.

3.15.3 Sequencing

Sequencing of 2µl of the purified DNA was performed on both the forward and reverse DNA strands separately using a BigDye terminator as described below:

3.15.3.1 Sequencing reaction

5X buffer	2.075µl
BigDye	0.500µl
Sterile distilled water	5.105µl
Primer forward or reverse (5µM)	0.320µl
	<hr/>
	8.000µl
PCR products	2.000µl
	<hr/>
Total	10.000µl

The reaction was amplified under the following conditions: one cycle of 96°C for 1 minute, followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

3.15.3.2 Clean up procedure for amplified products

The amplified products were cleaned up, denatured and dried before sequenced using an ABI Prism 3130X DNA sequencer (Applied Biosystems, USA).

3.15.4 Editing and analysis of the sequences generated

Consensus sequences for each of the samples were generated using DNASTar programme (Laser gene, USA; Version 7). The sequences obtained were submitted to the *H. pylori* MLST data base ([Http://pubmlst.org/Helicobacter](http://pubmlst.org/Helicobacter)) for allele and sequence identification. The FASTA format of the concatenated sequences of each sample was aligned and imported into MEGA version 5 (MEGA 5) and computed for phylogeny.

3.15.4.1 Allele and sequence type identification

Isolates MLST sequences data were queried against the global Helicobacter MLST database and alleles and sequence type (ST) Profiles assigned to individual isolates (assigned alleles and ST profiles through the stated MLST method).

3.15.4.2 Nucleotide analyses

The calculation of the ratio of synonymous to non-synonymous changes (dn/ds) was done using START2 (Sequence Type Analysis and

Recombinational Tests Version 2) tool which uses the method of Nei and Gojobori to estimate parameters [255].

3.15.4.3 Comparative analysis

For comparative analysis, 71 strains from Africa, 33 from Spain and 24 strains from Japan making 128 unique STs were downloaded from Helicobacter MLST web site ([Http://pubmlst.org/Helicobacter](http://pubmlst.org/Helicobacter)).

Concatenated sequences were aligned and imported into MEGA 5 [256].

The evolutionary history was inferred using the Neighbor-Joining method.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) was shown next to the branches.

3.15.4.4 Admixture and no-admixture models of *H. pylori* populations using STRUCTURE.

To determine the relatedness of the Gambian *H. pylori* to the previously studied strains from elsewhere, 170 strains from elsewhere were randomly selected from the public MLST data base (table 6.5). The non-admixture and admixture models of the program STRUCTURE V2.3.4 [257] were used to assign concatenated sequences of individual strains to previously identified bacterial populations. The highest value (K) that gave consistent clustering between individual runs was 4 (K=4).

3.16 Comparative statistical methods

3.16.1 Comparison of DNAs from culture and directly from biopsies

Percentage agreement was calculated to compare *H. pylori* genotypes obtained by PCR performed directly on gastric biopsies with the genotypes obtained by PCR of DNA extracted from bacteria cultured. In addition, the kappa statistic which allows for chance agreement (kappa=0 corresponds to no agreement beyond that expected by chance and kappa=1 represents perfect agreement) was reported.

3.16.2 Prevalence of *H. pylori* genotypes in different age groups

The prevalence of genotypes within different age categories was described. The null hypothesis of no association between prevalence and age was tested using Fisher's exact test and the differences among groups were tested using Fisher's exact test.

3.16.3 Prevalence of infection with single or multiple strains

The prevalences of infection with single vs. multiple strains was assessed. For the *cagA* gene, for example, the occurrence of *cagA* positive, *cag* empty site and mixed (*cagA* positive and *cagPAI* negative) infections was noted. Prevalences were compared between disease groups and p-values were determined using Fisher's exact test.

3.16.4 Antibiotic resistance in males vs females and between age groups

Comparisons of antimicrobial resistance in strains from children vs adults, males vs females, and distribution of *rdxA* nonsense mutations in different groups of strains were determined using Fisher's exact test; a P-value of <0.05 was considered significant.

Chapter 4 Prevalence of *Helicobacter pylori* virulent genotypes: PCR-based genotyping of *Helicobacter pylori* of Gambian children and adults directly from biopsy specimens and bacterial cultures

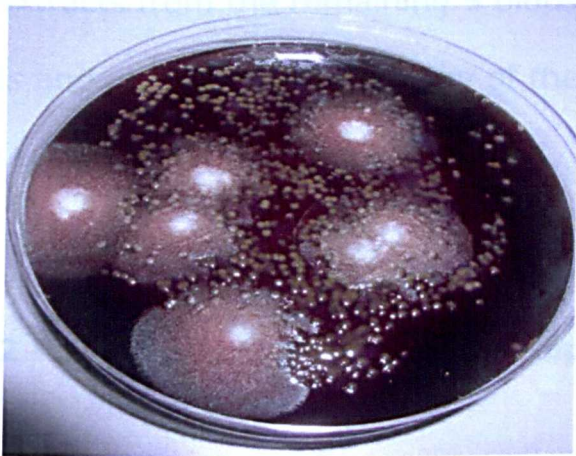
4.1 Introduction

H. pylori, because it is a fastidious micro-aerobic bacterium, it is technically difficult to grow and maintain for molecular biologic research in poorly resourced laboratories in Africa. These challenges coupled with the uniqueness of genotypes of African strains and special features of human physiology and environment in this continent limit our understanding of the spectrum of *H. pylori*-associated diseases and how this is affected by bacterial genotype in Africa [62,209]. So extensive efforts have been made to determine an optimum method for PCR-based genotyping of *H. pylori* [258-261] with various success rates. The methods used thus far to detect *H. pylori* infection such as stool antigen [262] and PCR restriction analysis using an RNA polymerase gene (*rpoB*) [259] were either found to be inadequate in detecting *H. pylori* virulent genes or experimentally too complex for routine purposes. To more effectively investigate prevalence of *H. pylori* genotypes and their influence on associated diseases in a West African setting, this chapter sought to determine an optimum method for PCR-based genotyping of *H. pylori* in The Gambia, and by extension determine the prevalence of *H. pylori* and *H. pylori* genotypes associated with the development of gastro-duodenal diseases in The Gambia, West Africa.

4.2 Results

A total of 169 biopsy samples from 169 subjects between the ages of 9 to 80 years, and 21 from young children between 18 to 31 months were investigated for *H. pylori* infections by both culture and PCR of DNA obtained directly from biopsies (figure 3.1). 89/169 (52.6%) biopsies cultured were initially identified as *H. pylori* culture positive. Pure *H. pylori* cultures were obtained from only 63 of them, but not the other 26, primarily because of overgrowth by contaminants despite inclusion of multiple antibiotics in the culture medium (figure 4.1) or bacterial cells failing to survive further subculture.

Figure 4.1 *H. pylori* selective media showing over growth of contaminants



4.3 Comparison of PCR and culture to detect *H. pylori*

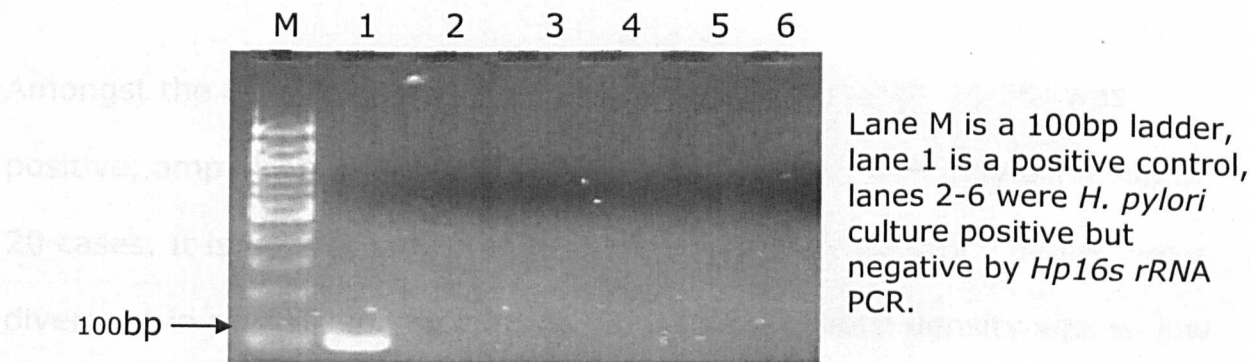
The same set of 169 biopsies cultured had DNAs extracted directly from the biopsy material. Direct PCR from these DNAs indicated that 164/169 (97%) were positive for *Hp16srRNA* (table 4.1).

Table 4.1 Comparison between culture results and direct PCR with *H. pylori* specific 16srRNA primer

Result	Culture	Direct by PCR
Positive	89 (52.6%)	164 (97.0%)
Negative	80	5
Total	169	169

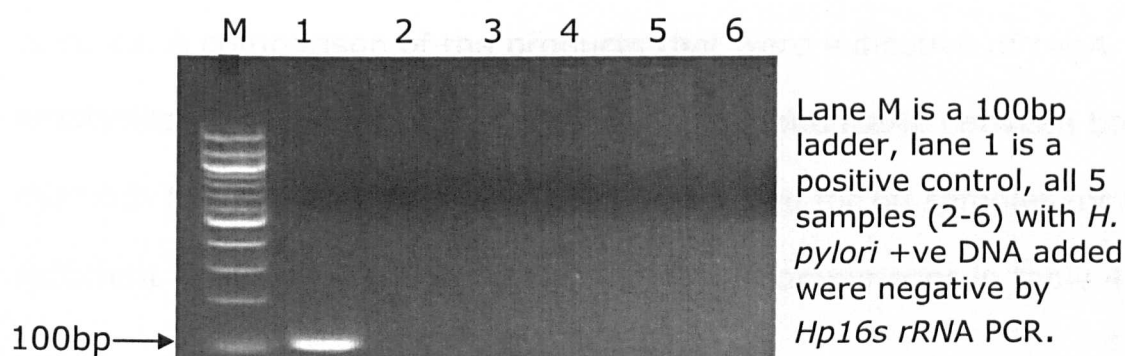
The DNA extracts from the remaining 5 biopsies were *H. pylori* negative with *Hp16s* and did not amplify for any of the genes tested, even though they were culture positive (figure 4.2).

Figure 4.2 PCR of 5 culture positive samples with *H. pylori* 16srRNA.



These five samples were tested for PCR inhibitors by addition of DNA from a known *H. pylori* positive sample. The samples which now contained *H. pylori* positive DNA were also all negative after PCR (figure 4.3). This implies the presence of a potent inhibitor.

Figure 4.3 PCR using *H. pylori* 16srRNA negative samples with *H. pylori* positive DNA



Of the 169 subjects between the ages 9-80 years, 121 biopsies from 121 subjects successfully amplified for virulence genes; among the 21 young children, 8 were *Hp16s* positive and pure *H. pylori* cultures were obtained successfully from six of them (figure 3.1). Direct PCR of the biopsies for virulent genes from the other 15 children were either negative (n=10) or not done (n=5).

Amongst the 80 culture negative adult subjects for whom *Hp16s* was positive, amplification of some or all virulence genes was only achieved in 20 cases. It is not yet known if these strains lacked virulence genes, were divergent in primer binding sequences, or the bacterial density was so low that amplification was possible only with the most general of primer

combinations, such as *Hp16s*. The remaining 60 samples did not show amplification for any of the genes tested despite a positive response to *Hp16s*.

4.4 Comparison of genotypes between PCR on bacterial cultures and direct PCR on biopsy material

Virulence gene data were obtained by direct PCR from both biopsies and cultures. A comparison of the products that were indicative of *cagA*, *cag* emptysite, *vacAs* alleles, *vacAm* alleles, *iceA1* and *iceA2* between both methods for detecting *H. pylori* was summarized for 60 samples for which sufficient amplified DNA was obtained for the comparisons in table 4.2.

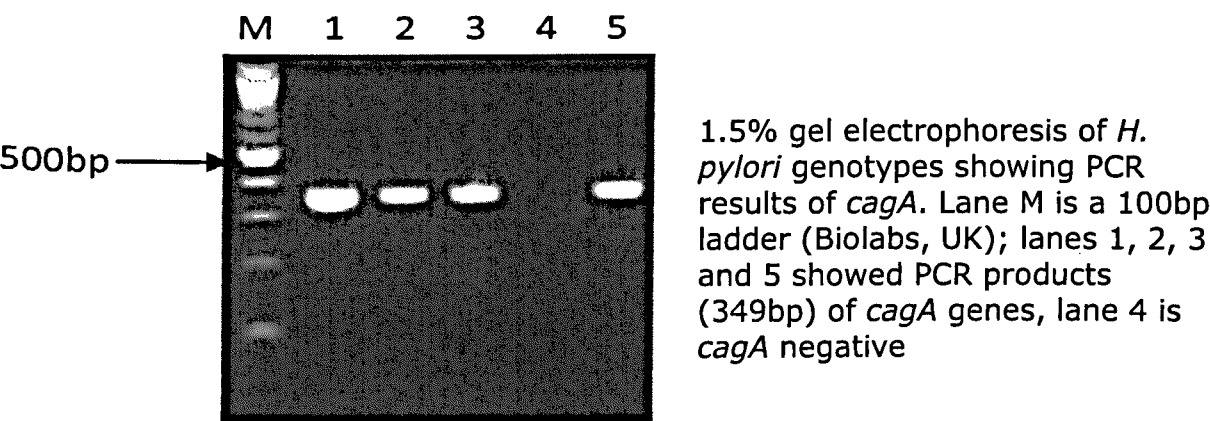
Table 4.2 Comparison of DNA from culture and tissue biopsy

	Culture (60)		Biopsy (60)		Failure to amplify DNA from biopsy material	
genotype	no	%	no	%	no	%
<i>cagA</i> status					7	11.7
<i>cagA</i> ⁺	37	61.7	35	58.3		
<i>cagA</i> ⁻	14	23.3	13	21.7		
<i>cagA</i> ⁺ and <i>cagA</i> ⁻	9	15.0	5	8.3		
<i>s</i> allele					7	11.7
<i>s1</i>	39	65.0	39	65.0		
<i>s2</i>	14	23.3	14	23.3		
<i>s1</i> and <i>s2</i>	7	11.7	0	0		
<i>m</i> allele					10	16.7
<i>m1</i>	26	43.3	23	38.3		
<i>m2</i>	20	33.3	23	38.3		
<i>m1</i> and <i>m2</i>	14	23.3	4	6.7		
<i>iceA</i> allele					10	16.7
<i>iceA1</i>	8	13.3	6	10		
<i>iceA2</i>	46	76.7	14	23.3		
<i>iceA1</i> and <i>iceA2</i>	6	10	30	50		

4.5 Detection of *cagA* using biopsy material and culture

The proportion of samples that were *cagA*^{+ve} (figure 4.4) with DNA from biopsies and from culture was similar, 58.3% and 61.7% respectively (table 4.2).

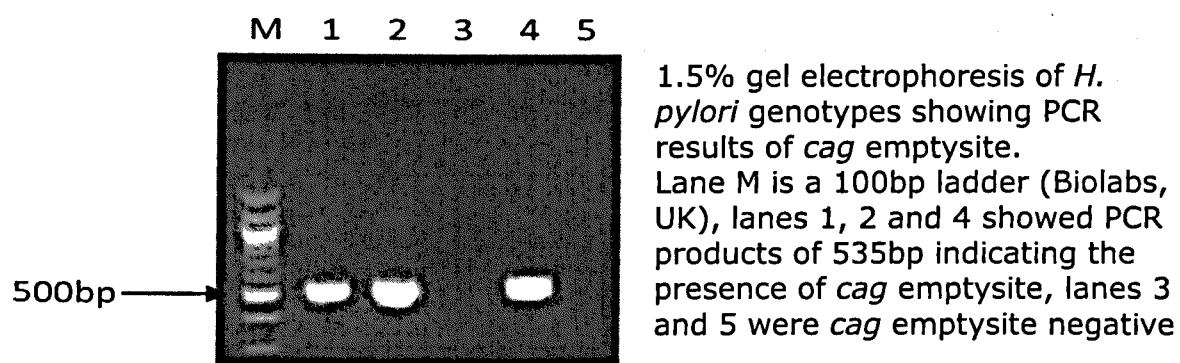
Figure 4.4 PCR inferred results of *cagA* gene



4.6 Detection of *cag empty site* using biopsy material and culture

For the *cag empty site*, the proportions of samples that were *cag empty site* positive (figure 4.5) with DNA from culture isolates and tissue material were also similar, 23.3% and 21.7% respectively (table 4.2) but the presence of strains that were both *cagA* gene positive and *cagA* negative were higher in DNA from culture isolates than from tissue biopsies, 15% vs 8.3% respectively.

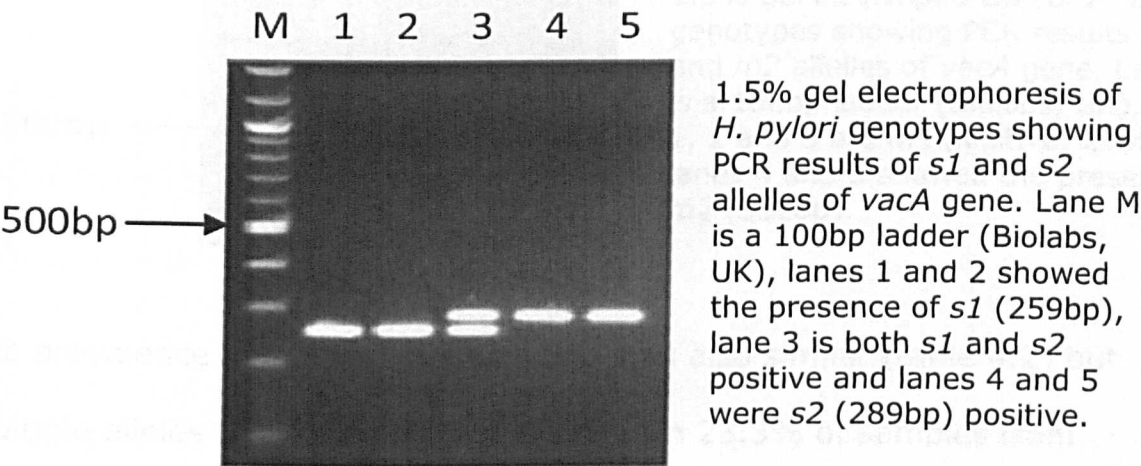
Figure 4.5 PCR inferred results of *cag empty site*.



4.7 Detection of signal region alleles (*s1,s2*) using DNA from biopsy material and culture

The presence of *s1* and/or *s2* allele was inferred when a product of the expected size, (259bp and/or 289bp) was obtained using appropriate primers (figure 4.6).

Figure 4.6 PCR inferred results of signal region of *vacA* gene.

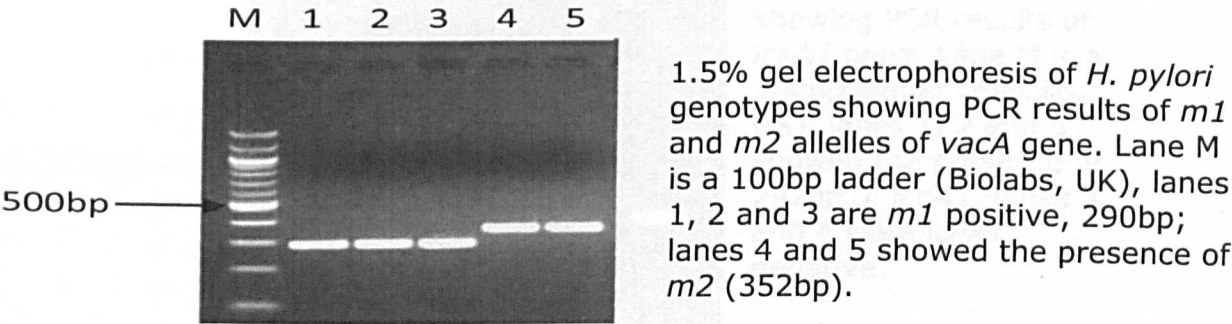


The success in amplification of *vacAs1/s2* (*s1* = toxigenic vs *s2* = non-toxigenic) from cultures and corresponding biopsies was similar (table 4.2 and 4.3). The prevalence of both *vacAs1* and *vacAs2* was 65% and 14% respectively. However, multiple alleles of *vacAs1/s2* were found only in 11.7% of samples from culture isolates and none from tissue biopsies (table 4.2).

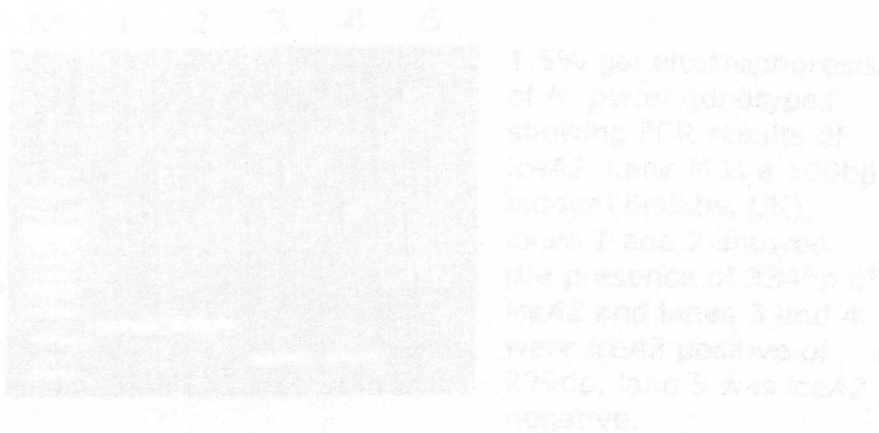
4.8 Detection of mid region alleles (*m1*, *m2*) using biopsy material and culture

For the *vacA* middle region (*m1* and/or *m2*), their presence were inferred when a product of the expected size, (290bp and/or 352bp respectively) were obtained using appropriate primers (figure 4.7).

Figure 4.7 PCR inferred results of mid region of *vacA* gene



The prevalence of *vacAm1* and *vacAm2* was also similar (table 4.2) but multiple alleles of *vacAm1/m2* were found in 23.3% of samples from culture isolates as oppose to 6.7% from tissue biopsies (table 4.2).



The prevalence of *iceA1* from culture isolates and corresponding biopsies was similar; 13.3% and 10% respectively. However, the presence of *iceA2* was abundant in culture isolates (76.7%) as opposed to 23.3% in

4.9 Detection of *iceA1* and *iceA2* using biopsy material and culture

The *iceA* gene has two allelic variants that yielded either 297bp (*iceA1*) or 229bp and 334bp (*iceA2*) products by PCR (figures 4.8 and 4.9).

Figure 4.8 PCR inferred results of *iceA1*

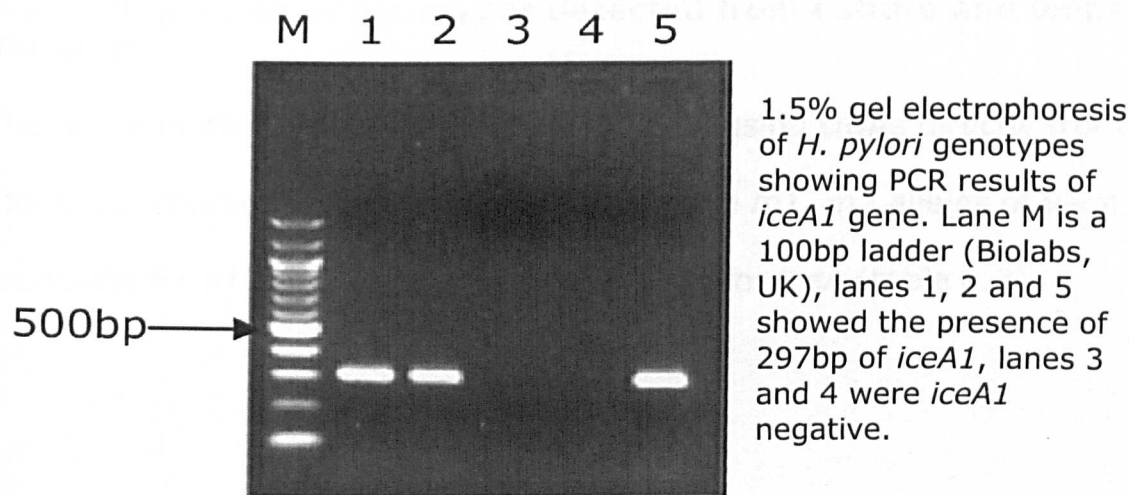
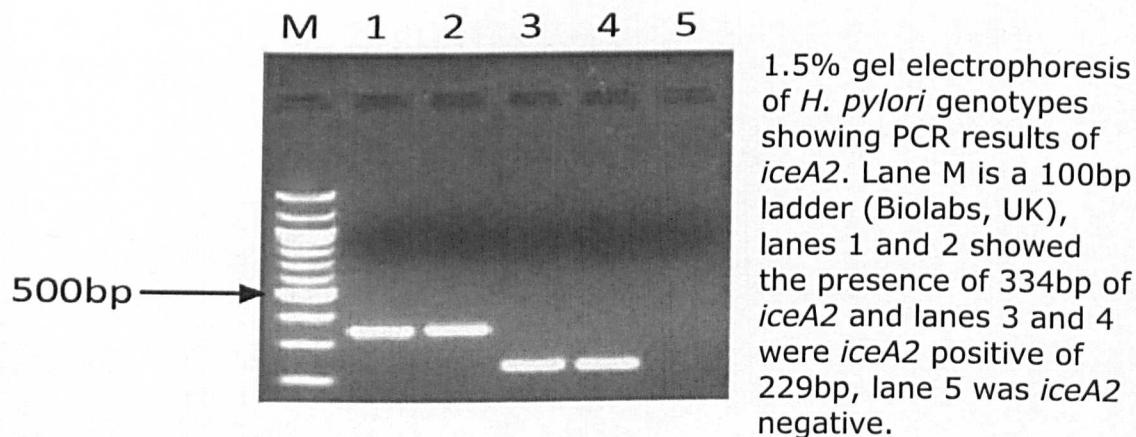


Figure 4.9 PCR inferred results of *iceA2*



The prevalence of *iceA1* from culture isolates and corresponding biopsies was similar; 13.3% and 10% respectively. However, the presence of *iceA2* was abundant in culture isolates (76.7%) as opposed to 23.3% in

the corresponding tissue biopsies. Multiple genes (*iceA1* and *iceA2*) were four times higher in tissue biopsies than the corresponding cultures; 50% vs 10% (table 4.2).

4.10 Comparison of genotypes detected from culture and biopsy material

The agreements between genotypes inferred using DNAs directly from these two sources was good for both *cagA* and *m1*, *m2* alleles of *vacA*, moderate for *s1*, *s2* alleles of *vacA*, and poor for *iceA* (table 4.3).

Table 4.3 Comparison of amplification of virulence genes between PCR on bacterial cultures and direct PCR on biopsy material

Culture	PCR on Biopsy				% Agreement (95% CI)	Kappa (95% CI)
	cagA+ n (%)	cag PAI empty site n (%)	cagA+ cag PAI empty site n (%)	No amplification with primer n (%)		
CagA+	31(52)	0(0)	3(5)	3(5)		
cag PAI empty site	0(0)	12(20)	0(0)	2(3)		
cagA+ cag PAI empty	4(7)	1(2)	2(3)	2(3)	84.9 (75.3,94.5)	0.70 (0.50,0.91)
	s1	s2	s1&s2	Missing		
s1	31(52)	4(7)	0(0)	5(8)		
s2	5(8)	9(15)	0(0)	1(2)		
s1&s2	3(5)	1(2)	0(0)	1(2)	75.5 (63.9,87.1)	0.45 (0.21,0.68)
	m1	m2	m1&m2	Missing		
m1	18(30)	1(2)	1(2)	6(10)		
m2	1(2)	17(28)	0(0)	2(3)		
m1&m2	4(7)	5(8)	3(5)	2(3)	76.0 (64.2,87.8)	0.62 (0.42,0.82)
	iceA1	iceA2	iceA1&2	Missing		
iceA1	5(8)	0(0)	1(2)	3(5)		
iceA2	1(2)	11(18)	29(48)	7(12)		
iceA1&2	0(0)	3(5)	0(0)	0(0)	32.0(19.1,44.9)	0.06(-0.05,0.16)

The poor agreement in the *iceA* analysis stemmed from the many classified only as *iceA2* by PCR from bacterial culture but *iceA1* and *iceA2* by biopsy which could have been due to the fact that certain bacterial strains in a mixed infection grew much better than others in culture. In direct PCR up to 16.7% of culture positive biopsies failed to amplify DNA for individual alleles.

The proportion of biopsies that were *cagA*⁺, the proportion of *vacAs1*, and *vacAm1*, and the proportion of mixed cultures from individual subjects varied with age.

All PCR results, including samples obtained from cultures and from direct PCR on biopsies (127 in total) are summarized in table 4.4.

		<i>iceA1</i>	<i>iceA2</i>	<i>iceA1/2</i>	Not amplified	P-value
Age						
0-10	12	1 (8.3)	2 (16.7)	0 (0)	0.103	
11-20	12	1 (8.3)	2 (16.7)	0 (0)		
21-30	12	1 (8.3)	2 (16.7)	0 (0)		
31-40	12	1 (8.3)	2 (16.7)	0 (0)		
41-50	12	1 (8.3)	2 (16.7)	0 (0)		
51-60	12	1 (8.3)	2 (16.7)	0 (0)		
61-70	12	1 (8.3)	2 (16.7)	0 (0)		
Total	127	10 (14.2)	41 (33.9)	17 (14.9)	0.065	

This data (table 4.4) is further illustrated in the bar charts below:

Table 4.4 Variation in frequency of alleles with age from samples obtained by PCR directly from biopsies or subcultured *H. pylori*.

Age (years)	Total	<i>cagA</i> ⁺	<i>cagA</i> ⁻	<i>cagA</i> ⁺ & <i>cagA</i> ⁻	Not amplified	P-value
1.5-2.5	6	1 (16.7)	5 (83.3)	0 (0)	0(0)	
9-29	54	35 (64.8)	10 (18.5)	8 (14.8)	1(1.8)	
30-40	31	22 (71.0)	2 (6.5)	6 (19.3)	1(3.2)	
41-59	24	15 (62.5)	4 (16.7)	4 (16.7)	1(4.2)	
>=60	12	2 (16.7)	5 (41.7)	5 (41.7)	0(0)	0.001
Total	127	75 (59)	26 (23)	23 (18.1)	3(2.4)	
Age (years)	Total	<i>s1</i>	<i>s2</i>	<i>s1&s2</i>	Not amplified	P-value
1.5-2.5	6	1 (16.7)	5 (83.3)	0 (0)	0(0)	
9-29	54	43 (79.6)	9 (16.7)	0 (0)	2(3.7)	
30-40	31	27 (87.1)	3 (9.7)	0 (0)	1(3.2)	
41-59	24	18 (75.0)	5 (20.8)	0 (0)	1(4.2)	
>=60	12	5 (41.7)	6 (50.0)	1 (8.3)	0(0)	<0.001
Total	127	94 (74)	28 (22)	1 (0.8)	4 (3.1)	
Age (years)	Total	<i>m1</i>	<i>m2</i>	<i>m1&m2</i>	Not amplified	P-value
1.5-2.5	6	1(16.7)	5 (83.3)	0 (0)	0 (0)	
9-29	54	25(46.3)	17 (31.5)	10 (18.5)	2 (3.7)	
30-40	31	16(51.6)	8 (25.8)	5 (16.1)	2 (6.5)	
41-59	24	12(50.0)	4 (16.7)	4 (16.7)	4 (16.7)	
>=60	12	2(16.7)	7 (58.3)	3 (25.0)	0 (0)	0.103
Total	127	56(44)	41 (32.3)	22 (17.3)	8 (6.3)	
Age (years)	Total	<i>iceA1</i>	<i>iceA2</i>	<i>iceA1&2</i>	Not amplified	P-value
1.5-2.5	6	3 (50.0)	1 (16.7)	2 (33.3)	0 (0)	
9-29	54	3 (5.6)	19 (35.2)	29 (53.7)	3 (5.6)	
30-40	31	6 (19.3)	7 (22.6)	15 (48.4)	3 (9.7)	
41-59	24	4 (16.7)	10 (41.7)	7 (29.2)	3 (12.5)	
>=60	12	2 (16.7)	6 (50.0)	4 (33.3)	0 (0)	0.065
Total	127	18 (14.2)	43 (33.9)	57 (44.9)	9 (7.1)	

This data (table 4.4) is further illustrated in the bar charts below:

Figure 4.10 virulent genes in various age groups

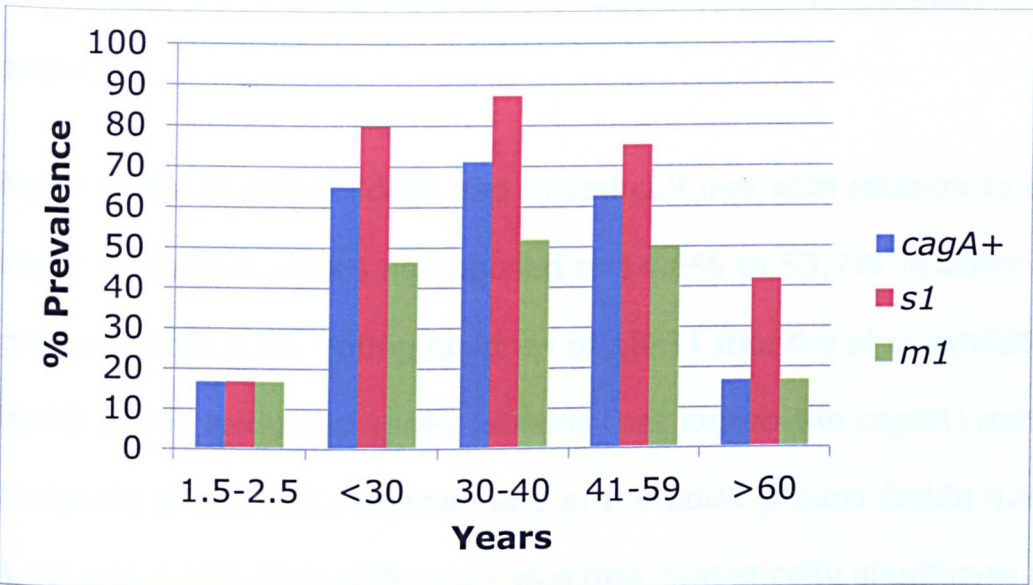
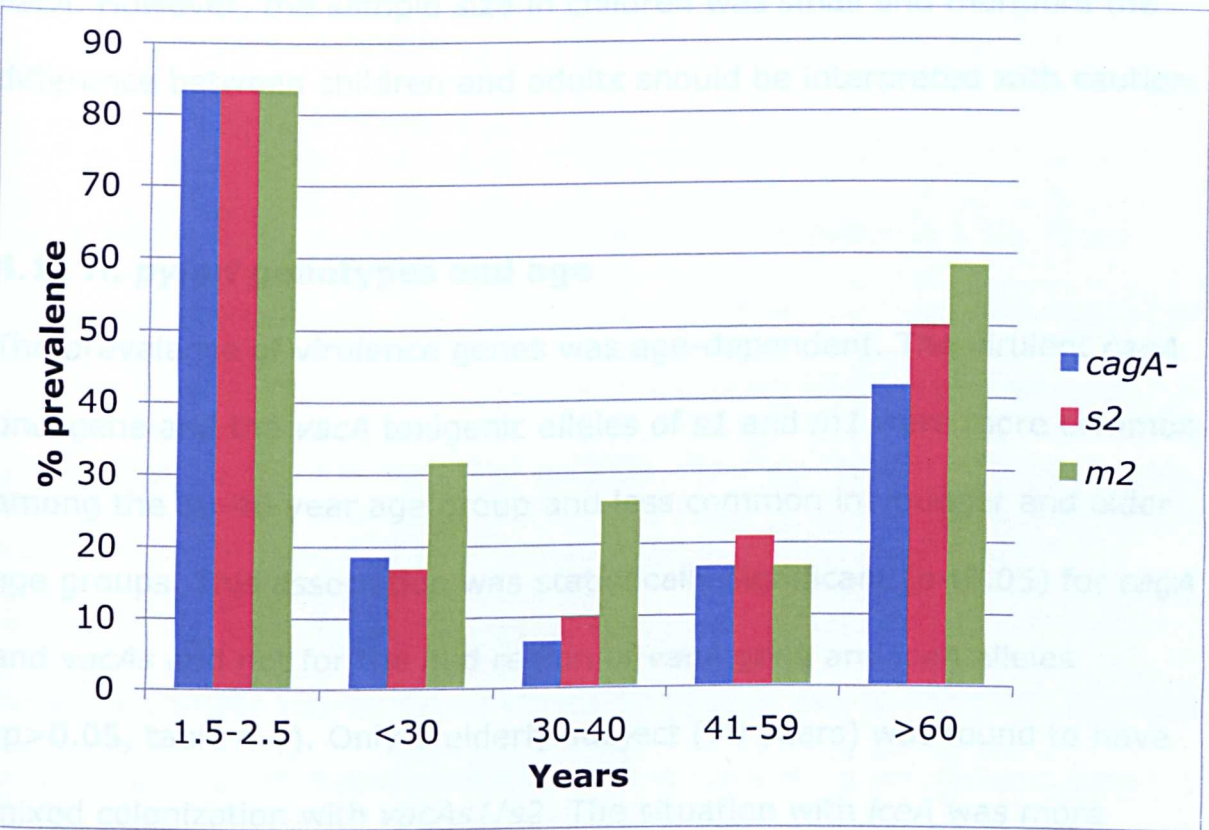


Figure 4.11 Non-virulent genes in various age groups



Where both culture from biopsy and PCR directly from biopsy were positive, only the results obtained from biopsy were included in this analysis.

None of the young children had mixed cultures with relation to *cagA*, *vacAs* or *vacAm* alleles as opposed to 14.8% to 53.7% in other age groups (table 4.4). Young children of 18-31 months also exhibited lower levels of the toxigenic genes (strains that lacked the *cagPAI* and the less toxigenic alleles (*s2,m2*) than any of the adult groups (table 4.4, figures 4.10 and 4.11). This difference was only statistically significant ($P \leq 0.02$) when isolates obtained from children were compared with those from adults aged less than 60 years for *cagA* and *s1* allele of *vacA*, and when compared with isolates from adults aged 41-59 years for *m1* region of *vacA*. However, the sample size in children was small and therefore the difference between children and adults should be interpreted with caution.

4.11 *H. pylori* genotypes and age

The prevalence of virulence genes was age-dependent. The virulent *cagA* oncogene and the *vacA* toxigenic alleles of *s1* and *m1* were more common among the 30-40 year age group and less common in younger and older age groups. This association was statistically significant ($p < 0.05$) for *cagA* and *vacAs* and not for the mid region of *vacA* gene and *iceA* alleles ($p > 0.05$, table 4.4). Only 1 elderly subject (70 years) was found to have mixed colonization with *vacAs1/s2*. The situation with *iceA* was more

complicated, with a large number of individuals exhibiting mixed *iceA1/iceA2* colonization.

4.12 Discussion

This chapter describes the prevalence of *H. pylori* genotypes in The Gambia and also describes the comparison between results obtained from direct PCR to detect *H. pylori* from gastric biopsies in West Africa, compared to PCR of bacterial isolates obtained from the same set of gastric biopsies. The present study establishes the prevalence of *H. pylori* infection in dyspeptic patients in The Gambia. The overall prevalence of *H. pylori* among the study patients was 97% by direct PCR and 53% by culture, although this varied with age. This prevalence is comparable with that of other developing countries.

Both PCR and culture techniques produced different success rates, as set out in tables 4.3-4.4, and both failed to detect *H. pylori* in a significant proportion of infections. The results presented here agreed with Park et al [263] in that direct PCR can produce inconsistent results, and tend to underestimate the prevalence of specific virulence factors (table 4.3-4.4). However, in this study, a good consistency of genotypes was detected between both techniques consistent with what was reported in a similar study by Chattopadhyay [258] where 83-97% of paired samples (biopsies vs cultures) had identical results for the various *H. pylori* virulent *cagA*

and *vacA* genes [258] but different from another study where only 32.8% of paired samples had identical results [264].

This data differs in that considerably greater difficulty in obtaining pure subcultures of *H. pylori* from gastric biopsies than Park, with a consequently higher failure rate was experienced. I have been involved in studies cultivating *H. pylori* from gastric biopsies from populations throughout the world, and it is my personal observation that sub-culture failure is a particular problem amongst West African isolates, as encountered in the present study. The reasons for this are not immediately apparent.

As a consequence of this problem, not all biopsies from which virulence factor DNA was amplified yielded a primary isolation of *H. pylori*, and there was a significant loss of isolates at subculture. PCR from subcultures gave higher rates of mixed colonization for *cagA* and *vacA* genes than direct PCR of biopsies, in contrast to the situation reported elsewhere with higher culture success rates [263]. In Park's study, 27% of cases had mixed infections with tissue DNA as compared to only 9% with bacterial DNA. In this study, the high numbers of mixed infections observed in cultures may have been due to artifact, either by enhancement of a minor strain from within the stomach, or due to modification of genome during culture as observed in a clinical study where different genotypes in a patient which may have developed from repeated sub-culturing [265].

This study showed that direct PCR produced more positive results, gave rise to fewer concerns about the development of artifact, and was more rapid and convenient.

The data also indicate that there may be PCR inhibitors or potent nucleases in some gastric biopsies. In this case 3% of biopsy samples were inhibited for *H. pylori* and also for all virulent genes and up to about 12% for various individual virulent genes (table 4.3). This is consistent with findings in similar studies [19,260,263]. However, the underestimation of *H. pylori* by PCR in this study was similar to the 8.4% reported in a study by Park [263] but less than the 42% reported in another study [261]. Their occasional presence and the underestimated prevalence of specific virulence factors by direct PCR illustrate that culture can be a useful complement to direct PCR for studies in which complete ascertainment of *H. pylori* virulence factor genotypes, including mixed colonization, is desired.

The study showed a difference in predominant genotype with subjects' age. In addition to adults of various age groups, this study also investigated children who were very young (18-31months) and were therefore closer to the time of colonisation. Young children produced isolates that were more likely to be *cagA*-ve, and *vacAs2m2*, in contrast to adults who were more likely to harbour *cagA*+ve *vacAs1m1* isolates. The studies that have so far characterized child isolates (mainly from

children older than those in this study) have shown marked geographical differences of genotypes. For example, studies in Brazil, Slovenia, North America, Korea and Japan [81-85] have shown that between 64-75% of all child isolates carried the virulent *s1* allele of *vacA* gene. 82% of Korean isolates [84] carried the *m1* allele of *vacA* gene and 94% of *cagA* gene which is similar to figures reported in Japan [81]. Similarly in Brazil [82], the majority of child isolates were *m1* allele positive. In contrast, a significant number of adults carried the more virulent genes of *cagA* and *vacA* in Portugal [87] than isolates from children. This is similar to figures reported in Israel [88] where only about 25% of children carried the virulent *cagA* gene and 65% of the avirulent *vacA* alleles of *s2m2*. In this study, children were also less likely to have mixed populations of *H. pylori* strains, which may relate to children aged 18 to 31 months being relatively recently colonized by *H. pylori*, compared to older individuals. The strains of *H. pylori* discovered in adult stomachs, at ages when typical *H. pylori* associated diseases develop, may be genotypically distinct from the original strains that first colonized young Gambian children. This could be due to recombination of the *H. pylori* genome over the course of decades [104,266] and/or re-exposure to novel strains, with more pathogenic strains circulating predominantly amongst adults.

4.13 Conclusion

The prevalence of *H. pylori* in The Gambia is high and comparable to figures reported in other developing countries. However, in order to

detect the range of bacterial genotypes harbored by individual patients, direct PCR proved slightly superior to isolation of *H. pylori* by biopsy culture in this study, but the techniques were complementary to each other, and the use of both together produced the most complete picture. Despite the lower success rate and greater cost of *H. pylori* culture relative to PCR directly from biopsies, culturing *H. pylori* is still important for antibiotic susceptibility tests that could guide therapy and other phenotypic tests such as bacterial adherence, *cagA* and *vacA* action on mammalian cells, expression of other colonization and virulence traits for which PCR alone is unsuitable. The use of PCR to detect genotypes has greatly improved on the detection of genotypes in this study. Young children of 18-31 months exhibited lower levels of the toxigenic genes (strains that lacked the *cagPAI* and the less toxigenic alleles (*s2,m2*) than any of the adult groups (table 4.4).

Chapter 5 Mixed Infection with *cagA* Positive and *cagA* Negative Strains of *Helicobacter pylori* Lowers Disease Burden in The Gambia.

5.1 Introduction

Earlier reports indicated high prevalence of *H. pylori* colonization, but a low frequency of *H. pylori*-associated disease in Africa [62,209,267], a phenomenon that was called the “African enigma” [62]. DNA sequencing of housekeeping and virulence genes have shown that different sets of genotypes predominate in different human populations [160]. Of particular interests have been *H. pylori*’s *cagA* oncogene and toxigenic *s1* and *m1* alleles of its *vacA* gene, which have been implicated in gastroduodenal diseases caused by this pathogen both in epidemiologic [268,269], experimental animal and cell culture infection [270]. This said, several studies from different world regions have not detected such an association [79,80,268], an outcome suggesting the possibility of other virulence-modulating factors.

Individuals can be colonized by either a single or multiple strains of *H. pylori*, and even colonization by what is initially a single strain can, over time, lead to the emergence of multiple *H. pylori* subpopulations, due variously to mutation or to genetic recombination either between duplicate sequences in the single strain’s genome or with DNAs from other transiently colonizing strains [268]. The prevalence of such mixed infections has been reported to vary (5-68%) [264,271-274], depending on geographical region, whether in a developed or developing country

(low and high overall infection risk, respectively), and probably also methods of analysis. The *H. pylori* virulence-associated vacuolating cytotoxin (*vacA*) and *cag* pathogenicity island (*cagPAI*) genes, and also the *cag* empty site in strains lacking the *cagPAI*, are typically found in only one copy per genome [105,274-276]. Accordingly, detection of both the *cagA* gene and the *cag* empty site, or of both *s1* and *s2* (signal sequence; at 5' end of gene) or both *m1* and *m2* (middle region) alleles of *vacA* in a biopsy or in pool of *H. pylori* from a person indicates mixed infection.

We speculated if having mixed infection might influence the risk of gastric disease; for example, if strains of different genotypes might occupy a broader range of niches in the stomach as has been seen during experimental infection [253] and thereby impact on clinical outcome. In this chapter, we investigated the genotypes of *H. pylori* in The Gambia and the relation of apparently single versus mixed infections to gastro-duodenal diseases. Biopsy samples used for this study were collected from only the antrum of subjects. Although our primary aim was to look at variation within single biopsies, using both antral and body biopsies would have been more optimal to determine *H. pylori* strains that colonize different regions of the stomach. However, some Gambian subjects were happy to allow research biopsies from different regions of the stomach. Others consented to a single additional research biopsy, which was taken from the gastric antrum, the preferential site for *H. pylori* colonisation

[62]. As we only had a complete set of antral biopsies, we therefore only included biopsies collected from the gastric antrum in this chapter.

5.2 Results

5.2.1 Gastroscopy results of all patients referred for endoscopy

Clinical data from the MRC Unit in The Gambia revealed that of 428 patients with gastric complaints investigated by gastric endoscopy between 2003-2008 (figure 3.1), 8 (1.9%) had gastric carcinoma, 20 (4.7%) and 15 (3.5%) had gastric and duodenal ulcers respectively, and that the others (89.9%) did not have such overt disease (diagnosed as non-ulcer dyspepsia (NUD) as shown in table 5.1.

Table 5.1 Endoscopy results of patients

Disease	No	%
Gastric ulcer	20	4.7
Duodenal ulcer	15	3.5
Gastric erosion	9	2.1
Gastric cancer	8	1.9
Gastritis (endoscopic appearance)*	256	59.8
Normal gastric tract appearance*	93	21.7
Oesophagitis*	20	4.7
Oesophageal ulcer*	4	0.9
Oesophageal cancer*	2	0.5
Duodenal diverticulitis*	1	0.2
Total	428	100

*Non-ulcerative gastric diseases (NUD) = gastritis, oesophagitis, oesophageal ulcer, oesophageal cancer, duodenal diverticulitis or normal appearance of gastric tract

5.2.2 Gastrosocopy results of study subjects

Endoscopic examination showed that of the 121 study subjects whose biopsies tested positive for *H. pylori* (figure 3.1, table 5.2), 11 had gastric ulcer (GU), 7 had duodenal ulcer (DU), 1 had both gastric and duodenal ulcers, 7 had gastric erosions (GE), 1 had gastric carcinoma (GC) and all other subjects (94) who presented with either abdominal pain or dyspepsia had no evidence or history of gastric or duodenal ulcers.

Table 5.2 Gastrosocopy results of 121 patients

Disease	Number	%
Non-ulcerative disease (NUD)	94	78
Gastric ulcer (GU)	11	9
Duodenal ulcer (DU)	7	6
Gastric erosion (GE)	7	6
Gastric & Duodenal ulcer (G+DU)	1	1
Gastric cancer (GC)	1	1
Total	121	100

Our previous data (Chapter 4) demonstrated that in The Gambia detection of mixed isolates in individual biopsies was best undertaken by PCR amplification directly from biopsy material rather than by bacterial culture (Secka O Gut 2011). Consequently, the analyses in this chapter were based only on DNAs amplified directly from biopsy samples.

5.2.3 Prevalence of *H. pylori* genotypes

One hundred and twenty one patients of the 169 study participants (figure 3.1) were inferred to be infected with *H. pylori* when DNAs extracted from their biopsies were tested by PCR for the presence of *H.*

pylori cagA gene and *cag* empty site. Seventy four biopsies (61.2%) were positive for the *cagA* gene only, 21 (17.4%) were positive for the *cag* empty site only and 23 (19%) were positive for both. In parallel we also tested for the *vacA* gene presence and allele types. In all, 93 of 121 (76.9%) were positive only for the *vacAs1* allele, 23 (19.0%) were positive only for the *vacAs2* allele and 1 (0.8%) was positive for both. Only *m1* or only *m2* alleles of *vacA* were detected in 55 (45.5%) and 36 (29.8%) of biopsies tested respectively; both *m1* and *m2* (mixed infections) were found in 22 (18.2%) biopsies and up to 6.6% of biopsy DNAs failed to amplify for individual alleles (table 5.3).

Table 5.3 Prevalence of *H. pylori* genotypes

<i>H. pylori</i> genotypes	n	%
<i>cagA</i> ⁺	74	61.2
<i>cagA</i> ⁻	21	17.4
<i>cagA</i> ⁺ & <i>cagA</i> ⁻	23	19.0
No amplification of <i>cagA</i> or <i>cag</i> empty site	3	2.5
<i>s1</i>	93	76.9
<i>s2</i>	23	19.0
<i>s1</i> & <i>s2</i>	1	0.8
no amplification of <i>s1</i> or <i>s2</i>	4	3.3
<i>m1</i>	55	45.5
<i>m2</i>	36	29.8
<i>m1</i> & <i>m2</i>	22	18.2
No amplification of <i>m1</i> or <i>m2</i>	8	6.6

5.2.4 Association between *H. pylori* genotypes

Of the 93 *H. pylori* strains that were positive only for *vacAs1*, 72 (77.4%) were *cagA* positive compared with only 1 (4.3%) *cagA* positive among the 23 strains that were positive only for *vacAs2*; most (16) of them contained the *cag* empty site allele only (table 5.3). Similarly, nearly all *s1m1* positive biopsies (92.5%) contained *cagA* genes, whereas none of those containing only *vacA s2m2* allele were *cagA* positive (table 5.4).

Table 5.4 Association of *vacA* with *cagA Helicobacter pylori* genotypes

	<i>cagA</i> ⁺		<i>cagA</i> ⁻		<i>cagA</i> ⁺ & <i>cagA</i> ⁻		Incomplete <i>cagA</i>		
<i>H. pylori</i> genotypes	n	(%)	n	(%)	n	(%)	n	(%)	Total
<i>s1m1</i>	49	(92.5)	1	(1.9)	3	(5.7)	0	(0)	53
<i>s1m2</i>	9	(50)	3	(16.7)	5	(27.8)	1	(5.6)	18
<i>s2m2</i>	0	(0)	16	(88.9)	2	(11.1)	0	(0)	18
<i>s2m1</i>	0	(0)	0	(0)	0	(0)	0	(0)	0
<i>s1m1m2</i>	12	(66.7)	0	(0)	6	(33.3)	0	(0)	18
<i>s1s2m1m2</i>	0	(0)	0	(0)	1	(100)	0	(0)	1
<i>s2m1m2</i>	0	(0)	0	(0)	3	(100)	0	(0)	3
Incomplete <i>VacA</i>	4	(40)	1	(10)	3	(30)	2	(20)	10

Incomplete *cagA*=*cagA* and *cag* empty site were not detected
Incomplete *vacA*=either *vacAs* or *vacAm* regions were not detected (4/10 *vacAs1* was detected & *vacAm* was missing, 2/10 *vacAs2* detected and *vacAm* missing, 2/10 *vacAm1* detected and *vacAs* missing and for 2/10 both *vacAs* and *vacAm* were missing).

5.2.5 Association of *H. pylori* virulence genes with upper gastric diseases

cagA positive *H. pylori* strains were found more frequently among study participants with gastroduodenal diseases than those with NUD: duodenal ulcers (6/7; 85.7%), gastric erosions (5/7, 71.4%), gastric ulcers (8/11, 73%); no overt gastric disease (53/94, 56.4%; (tables 5.5 and 5.8).

Table 5.5 Association between *cagA* genotypes and disease type

<i>cagA</i> status	DU		GC		GE		GU		GUDU		NUD		Total	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
<i>cagA</i> ⁺	6	(85.7)	1	(100)	5	(71.4)	8	(72.7)	1	(100)	53	(56.4)	74	(61.2)
<i>cagA</i> ⁻	1	(14.3)	0	(0)	2	(28.6)	3	(27.3)	0	(0)	15	(16.0)	21	(17.4)
<i>cagA</i> ⁺ & <i>cagA</i> ⁻	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	23	(24.5)	23	(19.0)
No amplification	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	3	(3.2)	3	(2.5)
Total	7	(5.8)	1	(0.8)	7	(5.8)	11	(9.1)	1	(0.8)	94	(77.7)	121	(100)

DU=duodenal ulcer, GC=gastric carcinoma, GE=gastric erosion, GU=gastric ulcer, GUDU=gastric ulcer and duodenal ulcer, NUD=Non-ulcerative dyspepsia.

5.2.6 *vacA* alleles and clinical outcome

Toxigenic *s1m1* alleles were found in 6 of the 11 (54.5%) patients diagnosed with gastric ulcer, 42.9%, 42.9% and 42.6% in those with duodenal ulcers, gastric erosions and NUD, respectively (table 5.6). The prevalence of *vacA* alleles were similar in the two groups of patients; overt disease vs. NUD. That is, no association was found between *vacA* alleles and clinical outcome ($p=0.94$, tables 5.6 and 5.7).

Table 5.6 Association between *vacA* genotypes and disease type

<i>vacA</i> status	DU*		GC*		GE*		GU*		GUDU*		NUD*		Total	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
<i>s1m1</i>	3	(42.9)	1	(100)	3	(42.9)	6	(54.5)	0	(0)	40	(42.6)	53	(43.8)
<i>s1m2</i>	1	(14.3)	0	(0)	2	(28.6)	0	(0)	0	(0)	15	(16.0)	18	(14.9)
<i>s2m2</i>	0	(0)	0	(0)	2	(28.6)	3	(27.3)	0	(0)	13	(13.8)	18	(14.9)
<i>s1m1m2</i>	3	(42.9)	0	(0)	0	(0)	1	(9.1)	0	(0)	14	(14.9)	18	(14.9)
<i>s1s2m1m2</i>	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	1	(1.1)	1	(0.8)
<i>s2m1m2</i>	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	3	(3.2)	3	(2.5)
§Incomplete <i>vacA</i>	0	(0)	0	(0)	0	(0)	1	(9.1)	1	(100)	8	(8.5)	10	(8.3)
Total	7		1		7		11		1		94		121	

*Du=duodenal ulcer; GC=gastric cancer; GE=gastric erosion; GU=gastric ulcer; GUDU=gastric and duodenal ulcers; NUD=non-ulcerative diseases

§ Incomplete *vacA*=either *vacAs* or *vacAm* regions were not detected (4/10 *vacAs1* was detected & *vacAm* was missing, 2/10 *vacAs2* detected and *vacAm* missing, 2/10 *vacAm1* detected and *vacAs* missing and for 2/10 both *vacAs* and *vacAm* were missing).

Table 5.7 Association between *vacA* genotypes and clinical outcome

<i>H. pylori</i> genotypes	Overt gastric disease		NUD	
	n	%	n	%
<i>s1m1</i>	13	48.1	40	42.5
<i>s1m2</i>	3	11.1	15	16.0
<i>s2m2</i>	5	18.5	13	13.8
<i>s1m1m2</i>	4	14.8	14	14.9
<i>s1s2m1m2</i>	0	0	1	1.1
<i>s2m1m2</i>	0	0	3	3.2
§Incomplete <i>vacA</i>	2	7.4	8	8.5
Total	27	100	94	100

§Incomplete *vacA*=either *vacAs* or *vacAm* regions were not detected (4/10 *vacAs1* was detected & *vacAm* was missing, 2/10 *vacAs2* detected and *vacAm* missing, 2/10 *vacAm1* detected and *vacAs* missing and for 2/10 both *vacAs* and *vacAm* were missing).

Overt gastric disease = DU=duodenal ulcer, GC=gastric carcinoma, GE=gastric erosion, GU=gastric ulcer, GUDU=gastric ulcer and duodenal ulcer

NUD = gastritis, oesophagitis, oesophageal ulcer, oesophageal cancer, duodenal diverticulitis or normal appearance of gastric tract

5.2.7 *cagA* status and clinical outcome

In the 27 patients with overt gastric disease, 77.8% were *cagA* positive compared to 56.4% of those with NUD (p-value=0.05, tables 5.5 and 5.8). Mixed colonisation (figure 5.1) was only found in the group of patients diagnosed with non-ulcerative dyspepsia (NUD).

Table 5.8 *cagA* and clinical outcome

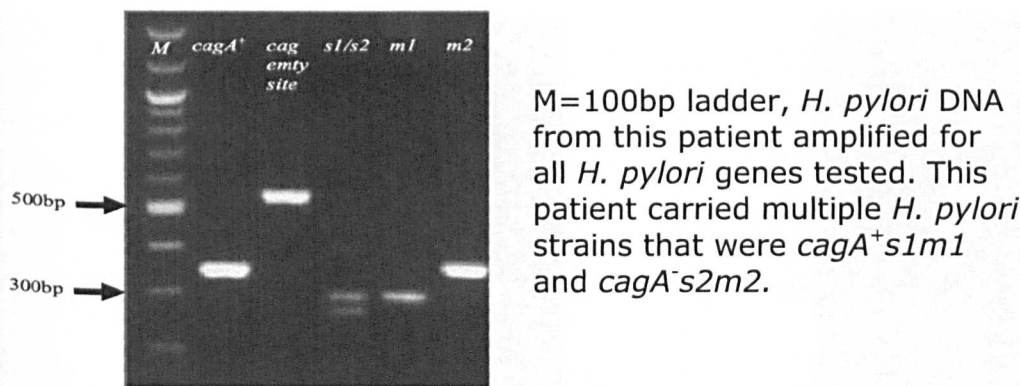
<i>cagA</i> status	Overt gastric disease		NUD	
	n	%	n	%
<i>cagA</i> ⁺	21	77.8	53	56.4
<i>cagA</i> ⁻	6	22.2	15	16.0
<i>cagA</i> ⁺ & <i>cagA</i> ⁻	0	0	23	24.5
no amplification	0	0	3	3.2
Total	27	100	94	100

Overt gastric disease = DU=duodenal ulcer, GC=gastric carcinoma, GE=gastric erosion, GU=gastric ulcer, GUDU=gastric ulcer and duodenal ulcer

NUD = gastritis, oesophagitis, oesophageal ulcer, oesophageal cancer, duodenal diverticulitis or normal appearance of gastric tract

Mixed infections were inferred based on three possible criteria (detecting both *cagA*⁺ and *cagA*⁻, detecting both *vacAs1* and *s2*, or detecting both *vacAm1* and *m2*). Twenty two or 23 patients had mixed infections, depending on which gene was analyzed (figure 5.1).

Figure 5.1 *H. pylori* multiple genotypes



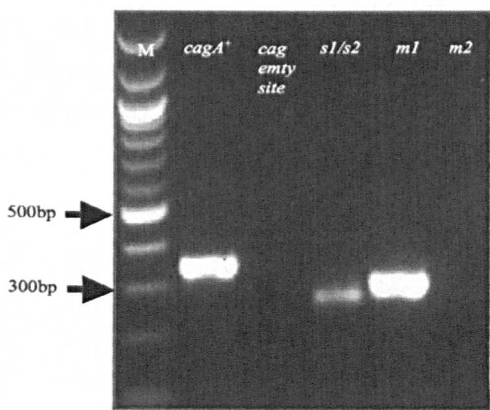
For *cagA* gene, 23 patients were considered to have mixed infections (*cagA* positive and *cagA* negative). Of these with *cagA* mixed infections, 10 were also positive for both *m1* and *m2* alleles of *vacA* and only one individual considered to have mixed infection for *cagA* gene was positive for both *s1s2* alleles.

Conversely, 12 individuals who were considered to have mixed infection for *vacAm1* and *m2* alleles were only positive for either *cagA* or *cag* empty site.

All 27 subjects with overt gastric diseases (table 5.8) were of uniform *cagA* status (that is, uniquely *cagA* gene positive or *cag* empty site

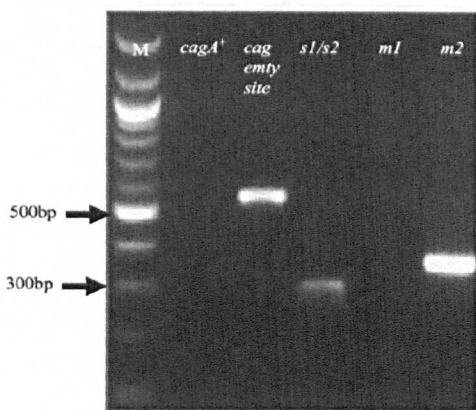
positive, figures 5.2 and 5.3), whereas only 72.3% (68/94) of NUD were of uniform status; the other 23 contained mixed (*cagA* positive, *cag* empty site positive) infections (figure 5.1).

Figure 5.2 Single *H. pylori* strain that is *cagA*⁺*s1m1*



M=100bp ladder, *H. pylori* DNA from this patient amplified for *cagA*, *s1* and *m1*. This patient carried a single *H. pylori* virulent strain (*cagA*⁺*s1m1*)

Figure 5.3 Single *H. pylori* strain that is *cagA*⁻*s2m2*



M=100bp ladder, *H. pylori* DNA from this patient amplified for *cag* emptysite, *s2* and *m2*. This patient carried a single *H. pylori* non-virulent strain (*cagA*⁻*s2m2*).

Three other biopsy samples did not give *cagA* gene or *cag* empty site amplification (table 5.8). This association between uniform *cagA* status and overt disease was statistically significant (p=0.002).

5.2.8 Association between age and clinical outcome

Higher prevalence of overt disease was found in those subjects that were over 40 years of age. Conversely subjects between 30-40 years had less overt diseases. However, across the entire trend of age distribution, no significant association was found between age and overt gastric disease

(24.5% <30 years, 12.5% 30-40 years and 27.8% >40 years; p-value=0.26, table 5.9).

Table 5.9 Association between age and clinical outcome

Age groups	Overt disease		NUD		Total
	n	(%)	n	(%)	
<30 years	13	(24.5)	40	(74.5)	53
30-40 years	4	(12.5)	28	(87.5)	32
>40years	10	(27.8)	26	(72.2)	36

p-value=0.26

5.2.9 Association between age and mixed infection

For mixed infections, subjects that were over 40 years were found to harbour strains that were both *cagA* positive and *cagA* negative. However, there was no association between age and frequency of mixed infection (15.1% <30 years, 18.8% 30-40 years, 25% >40 years; p-value=0.46, table 5.10).

Table 5.10 Association between age and mixed infection

Age groups	Mixed <i>cagA</i> (<i>cagA</i> + & <i>cagA</i> -)		Uniform <i>cagA</i> status (<i>cagA</i> + or <i>cagA</i> -)		Total
	n	(%)	n	(%)	
<30 years	8	(15.1)	45	(84.9)	53
30-40 years	6	(18.8)	26	(81.2)	32
>40years	9	(25.0)	27	(75.0)	36

p-value=0.46

5.3 Discussion

H. pylori infection is common in dyspeptic adults in The Gambia [19,277], as is typical of developing countries. The range of *H. pylori* genotypes implicated in overt gastroduodenal disease as opposed to benign colonization or possibly even beneficial carriage [269] had not been extensively investigated in Sub-Saharan Africa. Here we studied the distribution of *H. pylori*'s main virulence genes, *cagA* and toxigenic alleles of *vacA*, in The Gambia, and their possible associations with disease outcome.

The prevalence of gastroduodenal disease (10%) that we detected endoscopically is similar to that reported elsewhere in Sub-Saharan Africa [75] and may be lower than that in Europe and North America. In The Gambia, gastric carcinoma reported from patients who attended the only endoscopy unit in the country was 1.9% similar to what was reported in some sub-Saharan countries such as Ivory Coast (2%) and Cameroon (3%) but lower than the 6.6% reported in Kenya [75]. However, these estimated prevalences should be interpreted with caution as they may not be representative of the general population, but instead indicate the prevalence of disease among people with gastric complaints of sufficient severity to prompt diagnostic endoscopy.

We found that just over half of biopsy DNA (61.2%) carried the *cagA* gene (table 5.3) similar to the 61.6% found in Tunisia [278]; and that mixed infections (both *cagA* positive and *cagPAI* negative) were common. Multiple *vacA* genotypes (*m1* and *m2*) were also found in 22% of our study populations similar to the 18% reported in Korea [279] but higher than the 7% reported in Ethiopia [61]. High prevalences of mixed *vacA* genotypes (31%) were reported in a study in Tunisia [278].

We also found toxigenic *vacAs1* and *m1* alleles to be abundant in this study, but not universal (76.9% and 45.5%) similar to the 88.5% *s1* and 57% *m1* reported in Ethiopia [269]. In contrast, most *H. pylori* strains from Egypt carried *vacAs2* and *m2* alleles, (57.1% and 85.7%,

respectively) [269], whereas some 90% or more of strains in Japan, coastal China, and India carried *vacAs1* alleles [21,280,281]. *vacAm1* type alleles were also nearly universal in Japanese main island strains, whereas *vacAm2* strains were predominant in coastal China (81%) [281,282]. In this study, most of the *vacAs1* strains (77.4%) were *cagA* positive similar to what was observed in a study in South Africa (90%) [109].

Up to 6.6% of samples in this study failed to amplify for individual genes consistent with other findings [109,258,263], perhaps due to PCR inhibitors or potent nucleases in some gastric biopsies as also suggested previously [109,263,277].

Thus, our data reinforce conclusions that different *H. pylori* genotypes, especially types of genes or alleles implicated in virulent vs. benign infections, predominate in different human populations. Strains of the *vacA s1m2* type were most common in coastal China (81%), and also seemed to predominate in Southern Nigeria (74%) [170]; and both *s1m2* (20%) and *s2m2* (20%) allele types were abundant in South Africa [109]. This contrasts with the predominance of *vacA s1m1* strains found in our Gambian study participants. This apparent difference between Nigerian and Gambian strain genotypes could have several explanations, including the distance separating these two West African countries (>3000 km), or climatic differences (high rainfall and humidity in Southern Nigeria vs

aridity for much of the year in The Gambia). Such explanations would entail genetic divergence by random genetic drift and selection for adaptation to local conditions respectively.

Previous reports of a lower than expected prevalence of *H. pylori* associated disease in Africa [62,115,209] might in principle reflect the influence of bacterial and/or human genotypes, environment including other infections (e.g. parasitic infections that affect host response to *H. pylori*), normal gut microbiota, diet (including anti-oxidants, salt, spices) or likelihood of seriously ill persons being diagnosed and their cases entered in registries. In accord with this last explanation, have been suggestions that gastroduodenal disease is actually common in Africa, that there is no African enigma [79,80]. Until recently, life expectancy in Africa was relatively short compared to developed countries and because of this, the prevalence of gastric cancers and other gastroduodenal diseases which are diseases that develop in later life would be expected to be low. This coupled with poor maintenance of cancer case registries, under reporting of cases and limited access to health care facilities for most of the populations in Africa may have led to the concept of the "African enigma". As more populations get access to standard medical care and life expectancy increasing, prevalence rates of gastroduodenal diseases have been found to be similar to what was reported in industrialized countries [79].

The *cagA* gene and *vacAs1* and *m1* alleles are often linked to severe disease, and the *vacAs2* and *m2* alleles with more benign infections (gastritis only) in other populations. This is partially reflected in our results: with respect to *cagA* we did not find a “Gambian-*H. pylori* virulence gene” enigma similar to what was reported in Senegal [283]; disease associations with *vacA* were less clear cut in accord with reports from Sweden [284]. The possible effects of bacterial or human genetic and physiologic differences, food, history of other infections and other environmental and lifestyle factors, on outcomes of chronic *H. pylori* infections in sub-Saharan Africa merit further more detailed analyses.

Most important, was our finding that co-existence of *cagA* positive and *cagA* negative strains was significantly more common amongst patients with NUD than among those with overt disease, which suggests that mixed colonization is protective. In principle, protection against development of overt gastric disease might stem from simple competition – whereby carriage of a less virulent (*cagA*-negative) strain diminishes the vigour of growth of a coexisting virulent strain, thereby reducing its impact on host tissues. It is also possible that factors in *cagA*-negative strains that diminish the impact of virulence proteins such as CagA might predominate during *cagA*-positive and *cagA*-negative mixed infections [285]. Or, more generally, an increased complexity of immune responses during chronic infection by multiple divergent *H. pylori* strains might effectively diminish the inflammatory action of an individual virulent

strain, and thereby resultant pathology in host tissues, as noted with other infections [286-289]. In accord with this idea, the risk of developing overt disease seemed higher in subjects apparently colonized only with *cagA* negative *H. pylori*, than in those with mixed *cagA* positive and negative strains.

Further, the shift in the dominant strain seen in mixed infection due mainly to either recombination and/or mutation [186] may also help the bacteria to evade cellular responses by presenting antigenic variation and diversity [290].

Conversely, however, the presence of mixed infections might also stem from increased intrinsic host susceptibility to *H. pylori* infection and equally the development of a more severe clinical outcome [291-293].

5.4 Conclusion

This study has revealed frequent gastro-duodenal disease among Gambians with gastric complaints. Many strains carried *cagA*⁺ and *s1*, *m1* alleles of *vacA*, which are disease associated in many European and North American populations. Although *cagA* status was associated with disease in The Gambia, alleles of *vacA* were not. Comparison of our data with those from southern Nigeria pointed to a potentially significant difference in linkage of signal sequence (*s1* vs. *s2*) and middle region (*m1* vs. *m2*) alleles, which control the potency and tissue specificity of toxin action

respectively (*s1m1* most common in The Gambia, vs. *s1m2* most common in Nigeria). The possibility that such differences reflect selection for optimal genotypes or random genetic drift in these well-separated West African nations merit further study. We suggest that our most interesting finding is the significantly lower disease burden in Gambians infected with a mixture of *cag*-positive and *cag*-negative strains, relative to those containing only *cag*-positive or only *cag*-negative strains. The possibility that repeated exposure to colonisation by *H. pylori* would be beneficial in Sub-Saharan Africa and in developing countries more generally needs to be considered when developing more effective strategies for treating *H. pylori* infection and thus altering the risks of gastroduodenal disease [294].

Chapter 6 Population Genetic Analyses of *Helicobacter pylori* Isolates from Gambian Adults and Children.

6.1 Introduction

H. pylori is a genetically diverse Gram negative micro-aerophilic bacterial species that chronically infects some half of all humans worldwide, and in particular, most people in developing countries [295]. It is implicated in chronic gastritis, gastroduodenal ulcers and gastric cancer [70,296] and also increased risk of infection by diarrheal pathogens [141], infant malnutrition and growth faltering [251] in low income societies, although most infections are benign, and some may be beneficial [162,297]. The risk of infection resulting in overt disease is likely determined by *H. pylori* genotype in combination with other variables such as human genotype and physiology, nutrition and environmental factors.

H. pylori is usually acquired in childhood [298] and can persist for life unless eradicated by antibiotics [68]. A prevalence of $\geq 80\%$ is typical in developing countries [19,62,63,299], but has become far lower during the last century in industrialized countries (around 20%), probably due to dramatic improvements in hygiene and sanitation [100]. Transmission is predominantly intrafamilial with a low risk of adult infection in industrialized countries [89,101], whereas transmission within the local community is more frequent in developing countries, and often to adults as well as children, probably reflecting imperfect sanitation and hygiene in these societies.

Independent *H. pylori* isolates typically differ by some 2% or more in DNA sequence, allowing different strains to be distinguished readily by sequencing of one or more housekeeping genes [181]. This pathogen's great genetic diversity is also readily detected by the arbitrarily primed PCR (RAPD) method, wherein each strain yields a characteristic pattern of DNA fragments, different from those of nearly all other independent isolates [300]. In addition, DNA sequencing-based analyses (e.g. MultiLocus Sequence Typing, MLST) have shown that different sets of genotypes predominate in different human populations or geographic regions, such as East Asia and Western Europe [159,160]. This great diversity within and between populations can be ascribed to *H. pylori* having chronically infected humans for many thousands of years, with transmission being predominantly within families or local communities. This epidemiologic pattern allows considerable random genetic drift and selection for locally adapted genotypes. *H. pylori*'s genetic diversity is further enhanced by frequent mutation and recombination between strains during mixed infection [301]. MLST of seven housekeeping genes from strains from many parts of the world had identified seven *H. pylori* populations, designated hpEurope, hpEastAsia, hpAsia2, hpSahul, hpAfrica1, hpAfrica2 and hpNEAfrica [159,160,208]. Of particular importance to the analyses of Gambian *H. pylori* strains presented here, MLST analyses readily distinguished African strains (hpAfrica1, hpNEAfrica and hpAfrica2) from those of Europe and Asia and further subdivided

hpAfrica1 strains into two subpopulations: hspWAfrica (West) and hspSAfrica (South), the former also found in South Africa at low frequency. The distribution of hspWAfrica, hspSAfrica and hpNEAfrica populations may reflect the expansion of the Bantu people throughout the African continent over the last 4000 years from an ancestral homeland in or near present day Nigeria. The Bantu migrations from Central West Africa (present day Cameroon/Nigeria) structured the hpAfrica1 population into West African and South African subpopulations. In contrast to that, hpNEAfrica has mainly been isolated from Nilo-Saharan speakers.

The great majority of present Gambians come from indigenous West Africans, and of the predominant Mandinka, Wollof and Fulani linguistic groups, which are also abundant in nearby countries of Senegal, Guinea Bissau, Guinea Conakry and Mali. Most Gambians are Muslims, reflecting conversion of the resident population by Arab traders who began crossing the Sahara from North East Africa in the 8th century. Given the tendency of *H. pylori*'s populations to track with human host population, it is also noteworthy that for several centuries, The Gambia was also a major source of slaves taken to The Americas and also to Europe until the slave trade was abolished in 1807. This story implies that Gambian *H. pylori* strains may well have contributed significantly to *H. pylori*'s gene pool in The Americas and perhaps Europe as well. Indeed, a suggestion of West African admixture in European *H. pylori* had emerged in our early study of

a novel regulatory gene-linked insertion-deletion polymorphism (indel) in Spanish vs. Gambian *H. pylori* strains [302].

It is with this background that we carried out MLST of *H. pylori* strains from ethnic African adults and children in The Gambia. This *H. pylori* population is likely to be broadly representative of strains throughout much of West Africa, a relatively unstudied population, and as noted a contributor to the *H. pylori* gene pool in Europe and The America.

6.2 Patients

The patients selected for this study belonged to the following ethnic groups: Mandinka (19), Wollof (11), Jola (6), Fulani (5), Sarahule (4) Serere (1).

6.3 Sample choice for MLST

Samples were chosen simply according to successful subculture of individual *H. pylori* colonies. One or more single colonies were isolated from each of 44 patients and used for these analyses (figure 3.1). The 44 patients (23 male and 21 female) ranged in age from 18 months to 72 Years (mean 32 years) and had the following clinical manifestations: gastritis (23), normal gastric endoscopic appearances (6), gastric erosions (6), gastric ulcer (3), and oesophageal ulcer (1). Five of these patients were from malnourished children with enteropathy (ages 18-31 months, mean 19 months). Thirty three patients (75%) were from the Greater

Banjul (urban) Area (GBA) and 11 (25%) were from rural villages: [(LRR (6), WCr (4), NBR (1))].

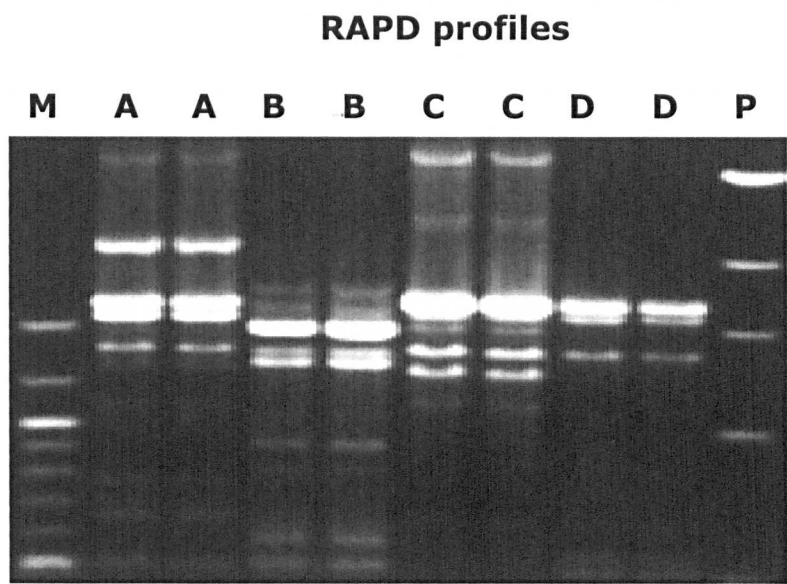
To look for genetic heterogeneity in the same stomach, several single colonies from each of two patients were tested by MLST. From one subject (14 years of age) with normal gastroduodenal tract appearance by endoscopy, seven colonies were analysed (4 antrum and 3 body). From the other subject (72 years of age), also with normal gastroduodenal tract appearance by endoscopy, 11 single colonies (6 antrum and 5 body) were sub-cultured and analysed.

6.4 Results

6.4.1 Allelic frequency and nucleotide analyses

DNAs from *H. pylori* strains from 44 Gambians (one strain/patient in 43 cases; three strains/patient in one case) yielded 42 unique MLST sequence types based on concatenated DNA sequences of seven house-keeping gene loci. There were four pairs of strains that yielded identical MLSTs. The two members of each pair were also identical by RAPD and virulent gene profiles (figure 6.1 and table 6.1). One pair was from consecutive unrelated patients, whose biopsy samples were taken on the same day. The other three pairs were from patients who had their biopsy samples taken between a week and two years apart.

Figure 6.1: RAPD profiles of samples with same MLST type



M=100bp DNA ladder, P= 1kb DNA ladder.
Paired letters indicate RAPD types of patients
with same MLST type

Table 6.1 Virulent genes of samples with same MLST types and RAPD profile

Lab number	<i>cagA</i>	<i>Empty site</i>	<i>m1</i>	<i>m2</i>	<i>s1s2</i>	MLST type	RAPD types
Hp42	+	-	+	-	1	2034	A
Hp112	+	-	+	-	1	2034	A
Hp71	-	+	-	+	2	2035	B
Hp72	-	+	-	+	2	2035	B
Hp250Ap	+	-	+	-	1	2062	C
Hp252Bp	+	-	+	-	1	2062	C
Hp268Bi	+	-	+	-	1	2040	D
Hp260AFi	+	-	+	-	1	2040	D

6.4.2 Allelic frequency

These exceptions aside, most alleles from the 46 strains occurred only once, although identical alleles were found in between 11 to 16 of the

strains, depending on the gene. Except for the four pairs of strains noted above, which were identical at all loci, no other pair of strains identical at one locus was identical at another of the seven loci tested (table 6.2).

Table 6.2 Alleles and sequence types of *H. pylori* isolates

Sample	House- keeping genes and allele type							MLST type
	<i>atpa</i>	<i>efp</i>	<i>muty</i>	<i>ppa</i>	<i>trpc</i>	<i>ureI</i>	<i>yphC</i>	
1*	1810	1703	1774	1761	1852	1891	1887	2034
2*	1810	1703	1774	1761	1852	1891	1887	2034
3*	1811	1656	1823	1762	1853	1892	1888	2035
4*	1811	1656	1823	1762	1853	1892	1888	2035
5	1812	1704	1824	1763	1854	1893	1889	2036
6	1813	1705	1825	1764	1855	1894	1890	2037
7	1814	1706	1826	1765	1856	1895	1891	2038
8	1815	1708	1828	1767	1858	1897	1893	2039
9*	1815	1729	1848	1774	1877	1919	1915	2040
10*	1815	1729	1848	1774	1877	1919	1915	2040
11	1816	1709	1829	1768	1859	1898	1894	2041
12	1817	1710	1830	1769	1860	1899	1895	2042
13	1818	1711	1831	1770	1861	1900	1896	2043
14	1818	1728	1847	1788	1876	1918	1914	2044
15	1819	1712	1832	1771	1862	1901	1897	2045
16	1820	1713	1833	1772	1863	1902	1898	2046
17	1820	1723	1774	1800	1888	211	1898	2047
18	1821	1714	1834	1773	1864	1903	1899	2048
19	1822	1715	1835	1774	1865	1904	1900	2049
20	1823	1716	1836	1775	1865	1905	1901	2050
21	1824	1717	1774	1776	1866	1906	1902	2051
22	1825	1703	1837	1777	1867	1907	1903	2052
23	1826	1718	1838	1778	1868	1908	1904	2053
24	1827	1719	1839	1779	1869	1909	1905	2054
25	1828	1720	1840	1780	1870	1910	1906	2055
26	1829	1721	1841	1781	1871	1911	1907	2056
27	1830	1722	1842	1782	1872	1912	1908	2057
28	1831	1723	1774	1783	229	1913	1909	2058
29	1832	1724	1843	1784	1873	1914	1910	2059
30	1832	1732	1850	1791	1880	199	1918	2060
31	1833	1725	1844	1785	1860	1915	1911	2061
32*	1834	1726	1845	1786	1874	1916	1912	2062
33*	1834	1726	1845	1786	1874	1916	1912	2062
34	1835	1727	1846	1787	1875	1917	1913	2063
35	1836	1730	1849	1789	1878	1912	1916	2064
36	1837	1731	1846	1790	1879	1920	1917	2065
37	1838	1733	1851	368	1881	1921	1898	2066
38	1839	1734	1852	1793	1881	1922	1919	2067
39	1840	1700	1853	1794	1882	1923	1920	2068
40	1841	1736	1854	1795	1883	1924	1921	2069
41	1842	1737	1855	1796	1884	1925	1922	2070
42	1843	1738	1846	1797	1885	1892	1923	2071
43	1844	1739	1856	1780	1870	1926	1924	2072
44	1845	1740	1823	1798	1886	1910	1925	2073
45	1846	1741	1857	1799	1887	1927	1926	2074
46	1847	1707	1827	1766	1857	1896	1892	2075

*indicates four pairs of samples with identical MLST types

The gene *trpC* had most samples with identical alleles (16) but allele 1774 of *mutY* was most frequent with 5 occurrences (10.9%, table 6.3). No deletions or insertions were found in this data set for all the analysed housekeeping gene fragments.

Table 6.3 Frequency of alleles

Gene	allele	Frequency	%
<i>atpa</i>	1810	2	4.3
	1811	2	4.3
	1815	3	6.5
	1818	2	4.3
	1820	2	4.3
	1832	2	4.3
	1834	2	4.3
<i>efp</i>	1656	2	4.3
	1703	3	6.5
	1723	2	4.3
	1726	2	4.3
	1729	2	4.3
<i>mutY</i>	1774	5	10.9
	1823	3	6.5
	1845	2	4.3
	1846	3	6.5
	1848	2	4.3
<i>ppa</i>	1761	2	4.3
	1762	2	4.3
	1774	3	6.5
	1780	2	4.3
	1786	2	4.3
<i>trpC</i>	1852	2	4.3
	1853	2	4.3
	1860	2	4.3
	1865	2	4.3
	1870	2	4.3
	1874	2	4.3
	1877	2	4.3
	1881	2	4.3
<i>ureI</i>	1891	2	4.3
	1892	3	6.5
	1910	2	4.3
	1912	2	4.3
	1916	2	4.3
	1919	2	4.3
<i>yphC</i>	1887	2	4.3
	1888	2	4.3
	1898	3	6.5
	1912	2	4.3
	1915	2	4.3

6.4.3 Analyses of selection

Mutation by nucleotide substitution in the coding region may result in no change in protein sequence (synonymous) or an amino acid change (non-synonymous). Estimating the diversity between Gambian isolates, we used the average number of synonymous and non-synonymous changes per site (d_s , d_n) of the 7 loci (table 6.4). The ratio of these two types in a population reflects genetic drift and selection operating on individual genes. Since all d_n/d_s values were close to zero; this indicates a high degree of selection to maintain amino acid sequence and function of the encoded protein. This is as expected for genes whose encoded proteins act within bacterial cells and provide important housekeeping functions. The most diverse gene was *trpC* (mean nucleotide level diversity 4.6%) and the least diverse was *ureI* (1.2%, table 6.4).

Table 6.4 Diversity between Gambian isolates

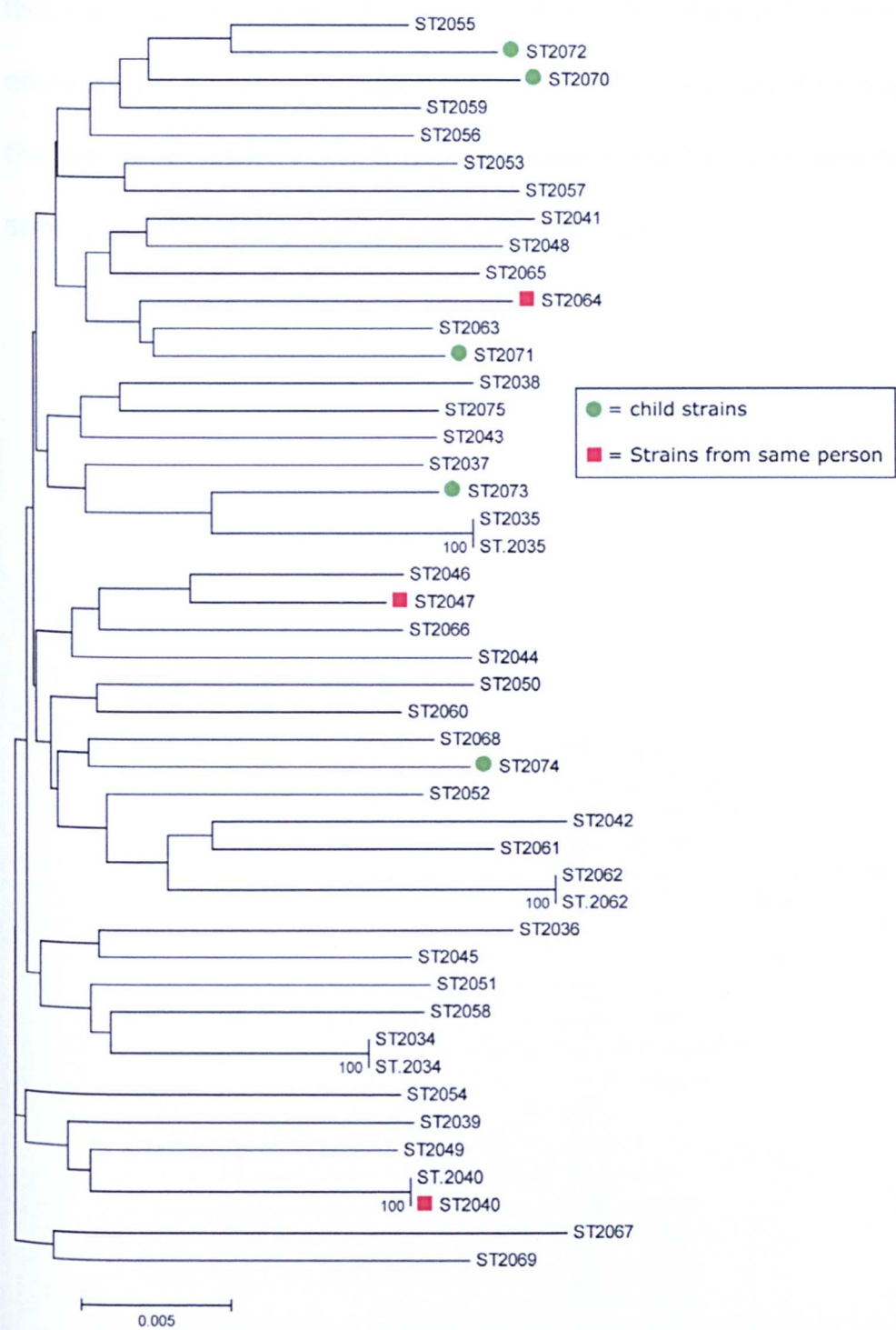
Locus	dn	ds	dn/ds	Diversity (%)
<i>atpA</i>	0.0006	0.1009	0.006	2.3
<i>efp</i>	0.0005	0.1059	0.0046	2.2
<i>mutY</i>	0.0098	0.1822	0.0536	4.5
<i>ppa</i>	0.0116	0.042	0.2761	2.1
<i>trpC</i>	0.0187	0.1513	0.1238	4.6
<i>ureI</i>	0.0052	0.0365	0.1428	1.2
<i>yphC</i>	0.0092	0.0924	0.0995	3.4
overall	0.0079	0.1016	0.1009	2.9

d_s and d_n : the average number of synonymous substitutions per synonymous site and non-synonymous substitutions per non-synonymous site, respectively.

6.4.4 Phylogenetic analysis

A phylogenetic tree formed using concatenated sequences of the seven housekeeping genes we used for MLST (figure 6.2) reflects a mean nucleotide level diversity of 2.9% (table 6.4).

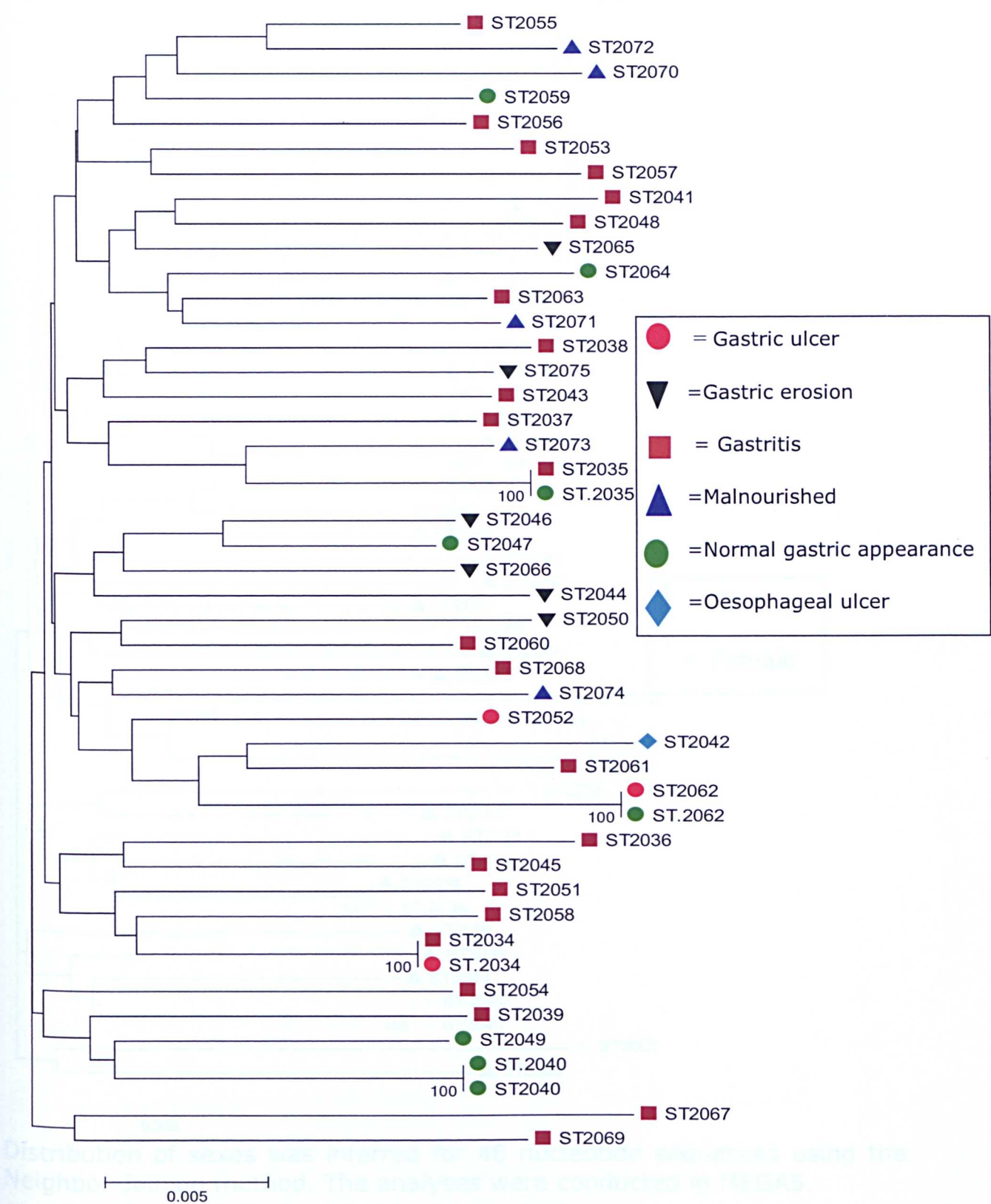
Figure 6.2 Evolutionary relationships of *H. pylori* strains from The Gambia



The evolutionary history was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5. The five strains from young children are identified with green balls. There was one subject with three different MLST types shown in red squares.

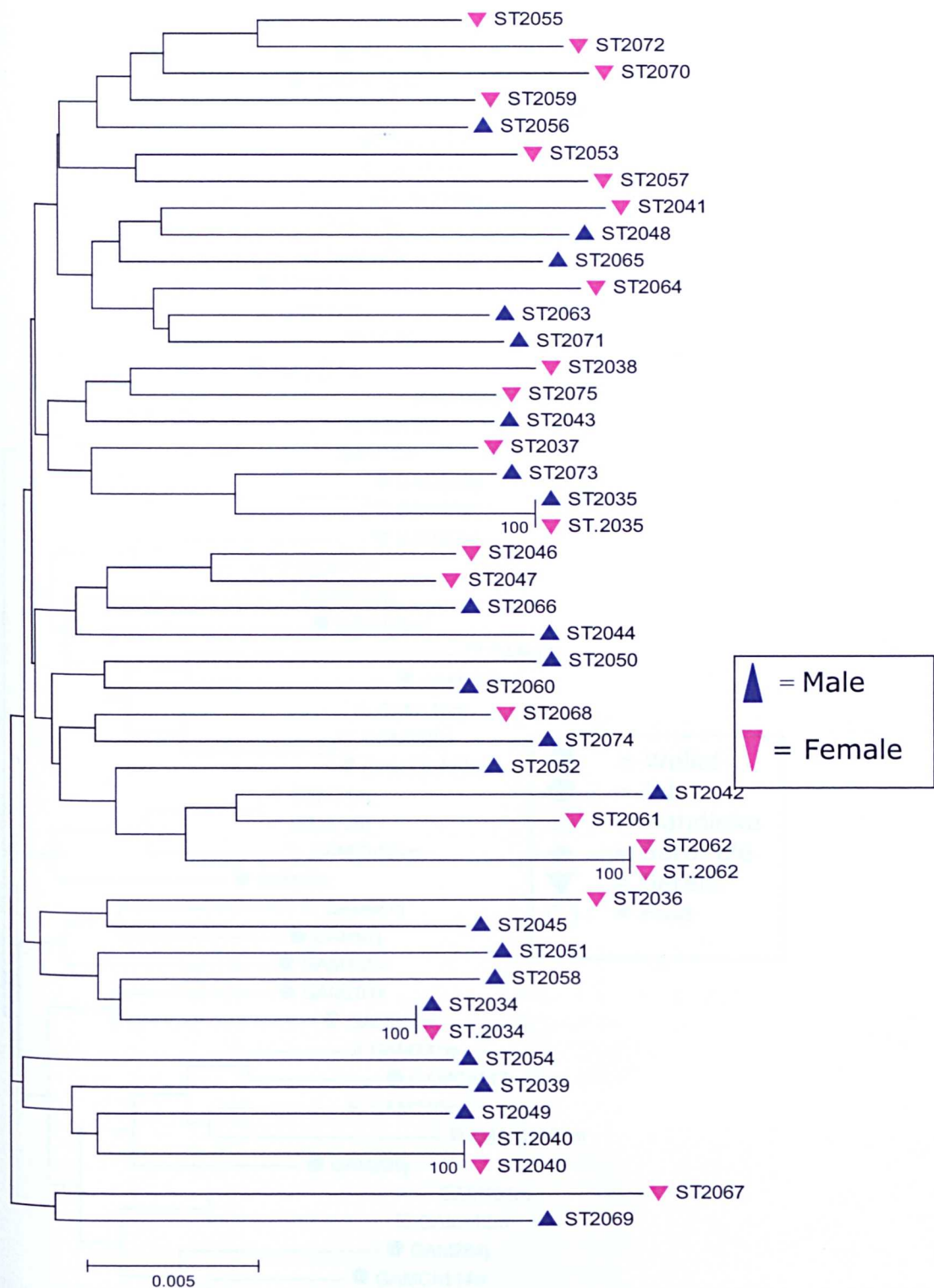
There was no evidence of association of particular clusters of strains in this tree (clades) with variables such as age of participant at time of endoscopy, endoscopic diagnosis, sex, tribe or district of residence within the Gambia (figures 6.3-6.6). However, *cagA*⁺ strains seemed to cluster separately from *cagA*⁻ (figures 6.7 and 6.8).

Figure 6.3 Distribution of diseases vs MLST types



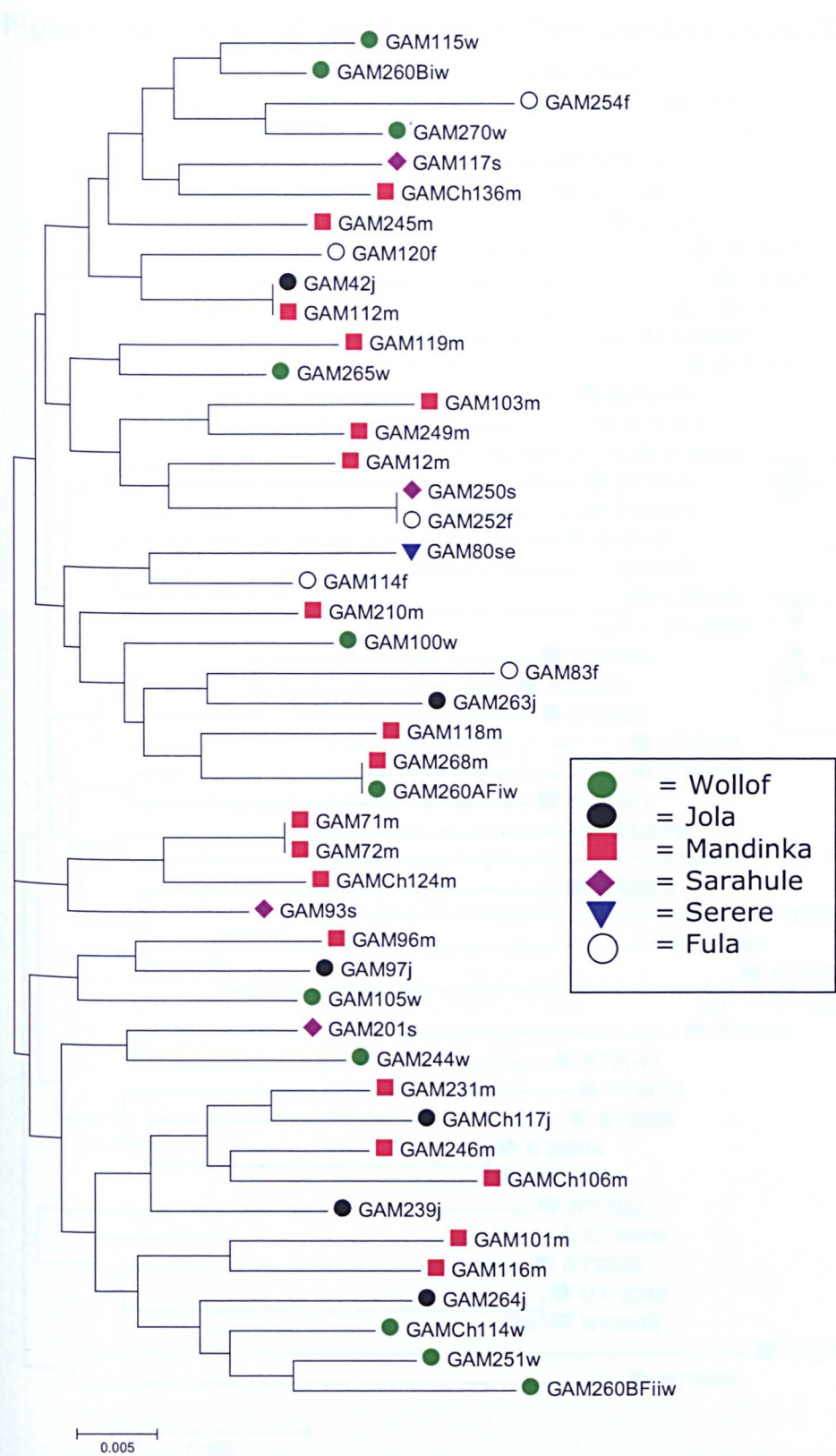
Distribution of diseases was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5.

Figure 6.4 Distribution of sex vs MLST types



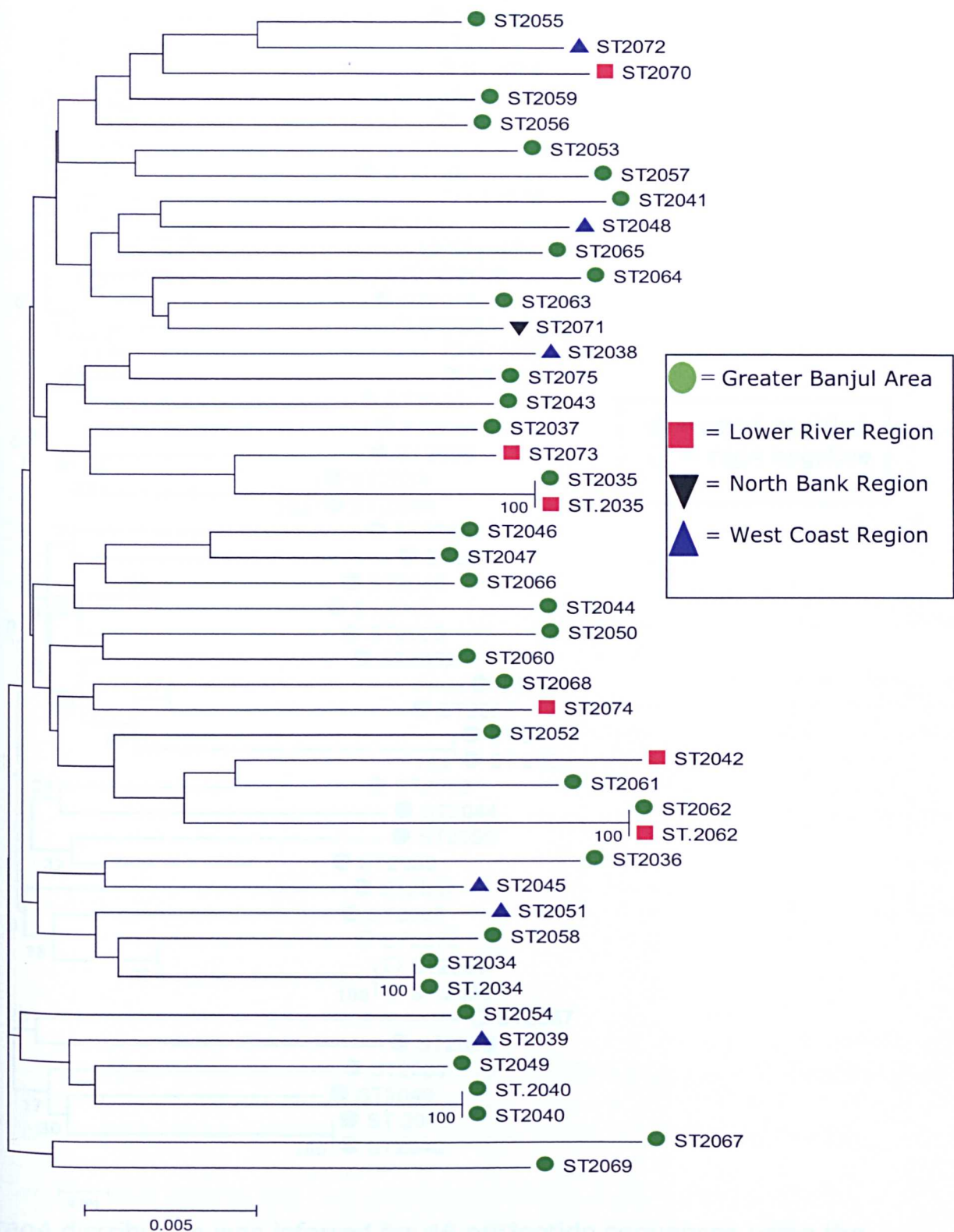
Distribution of sexes was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5.

Figure 6.5 Ethnicity and MLST types



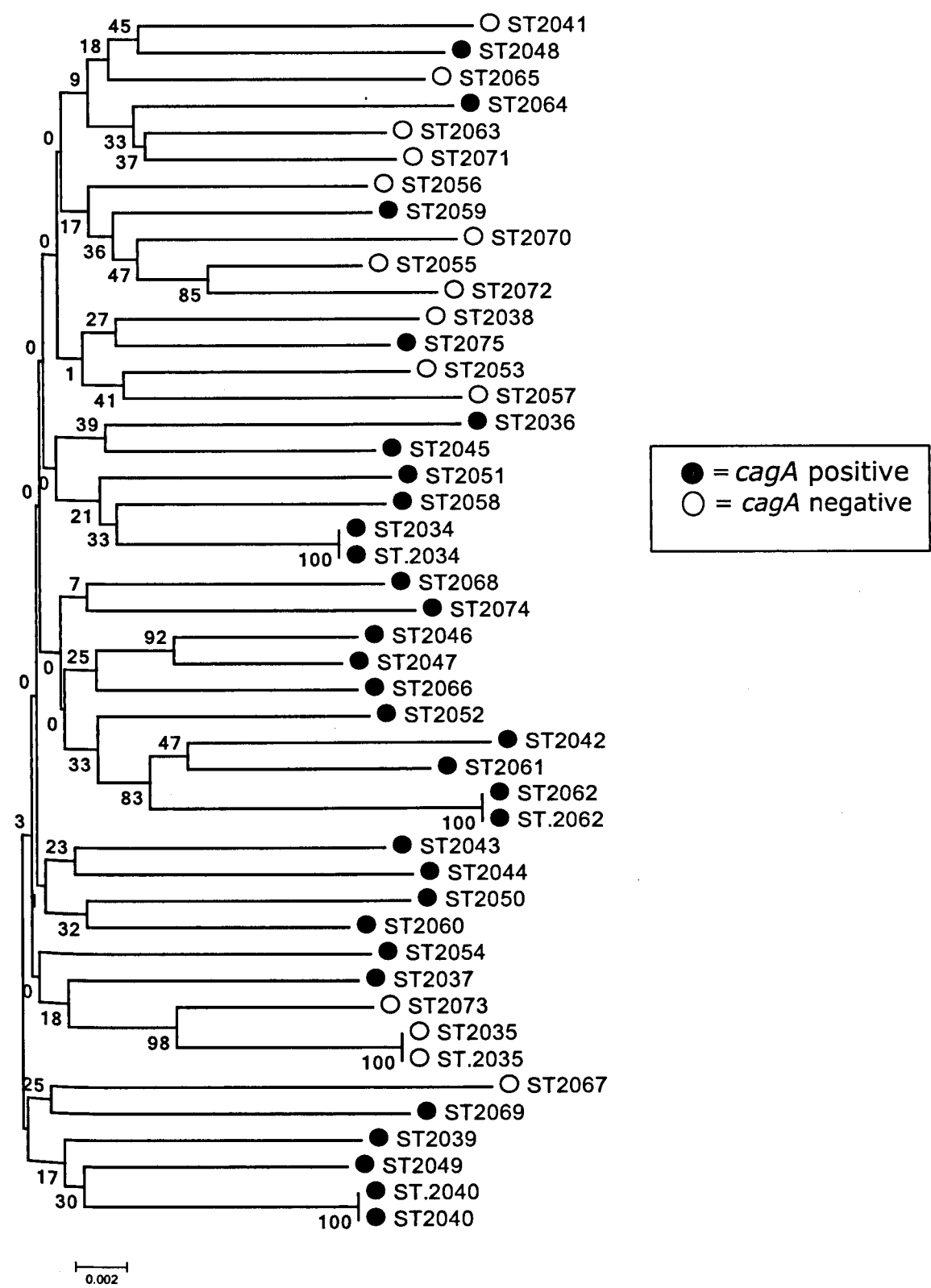
Ethnicity was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5.

Figure 6.6 Place of residence in The Gambia vs MLST types



Place of residence was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5.

Figure 6.7 *cagA* distribution of strains



cagA distribution was inferred for 46 nucleotide sequences using the Neighbor-Joining method. The analyses were conducted in MEGA5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

6.4.5 Cluster analysis of strains from other countries

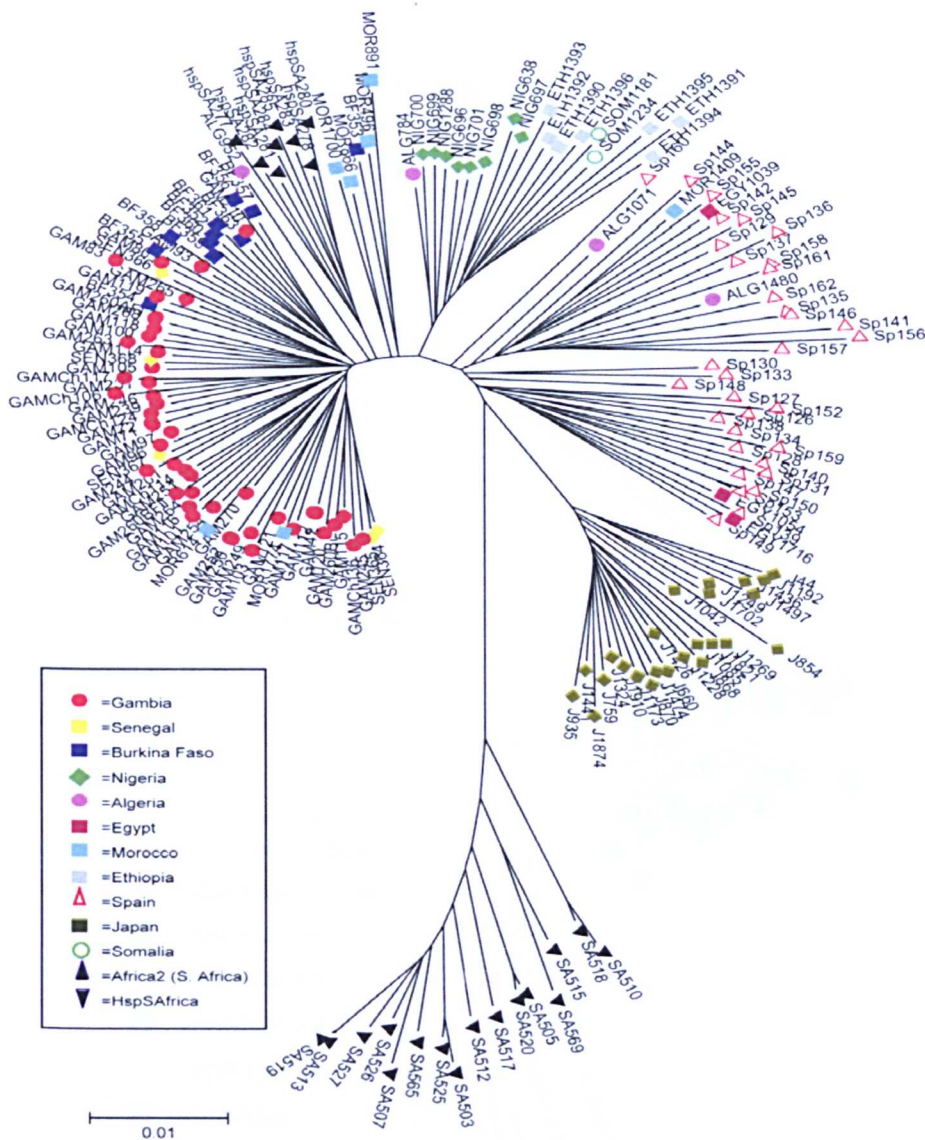
The Gambian strain data were compared with data from *H. pylori* strains selected randomly from other informative human populations (African, European and Asian, table 6.5) using neighbor joining and cluster analysis with both the admixture and no-admixture models of STRUCTURE.

Table 6.5 Selected *H. pylori* populations from MLST data base

Country	hpAfrica1	hpNEAfrica	hpEurope	hpEAsia	hpAfrica2	hpSahul	hpAsia2
Burkina Faso	11	0	0	0	0	0	0
Senegal	5	0	0	0	0	0	0
Nigeria	0	8	0	0	0	0	0
Algeria	1	1	2	0	0	0	0
Egypt	0	0	3	0	0	0	0
Ethiopia	0	7	0	0	0	0	0
Morocco	5	0	2	0	0	0	0
Somalia	0	2	0	0	0	0	0
Spain	0	0	33	0	0	0	0
South Africa	8	0	0	0	16	0	0
Japan	0	0	0	24	0	0	0
Finland	0	0	9	0	0	0	0
Estonia	0	0	11	0	0	0	0
Kazakhstan	0	0	0	0	0	0	1
Malaysia	0	0	0	0	0	0	1
Philippines	0	0	0	0	0	0	3
Thailand	0	0	0	0	0	0	4
Papua NewGuinea	0	0	0	0	0	7	0
Australia	0	0	0	0	0	5	0
Netherlands	0	0	0	0	0	0	1
Total	30	18	60	24	16	12	10

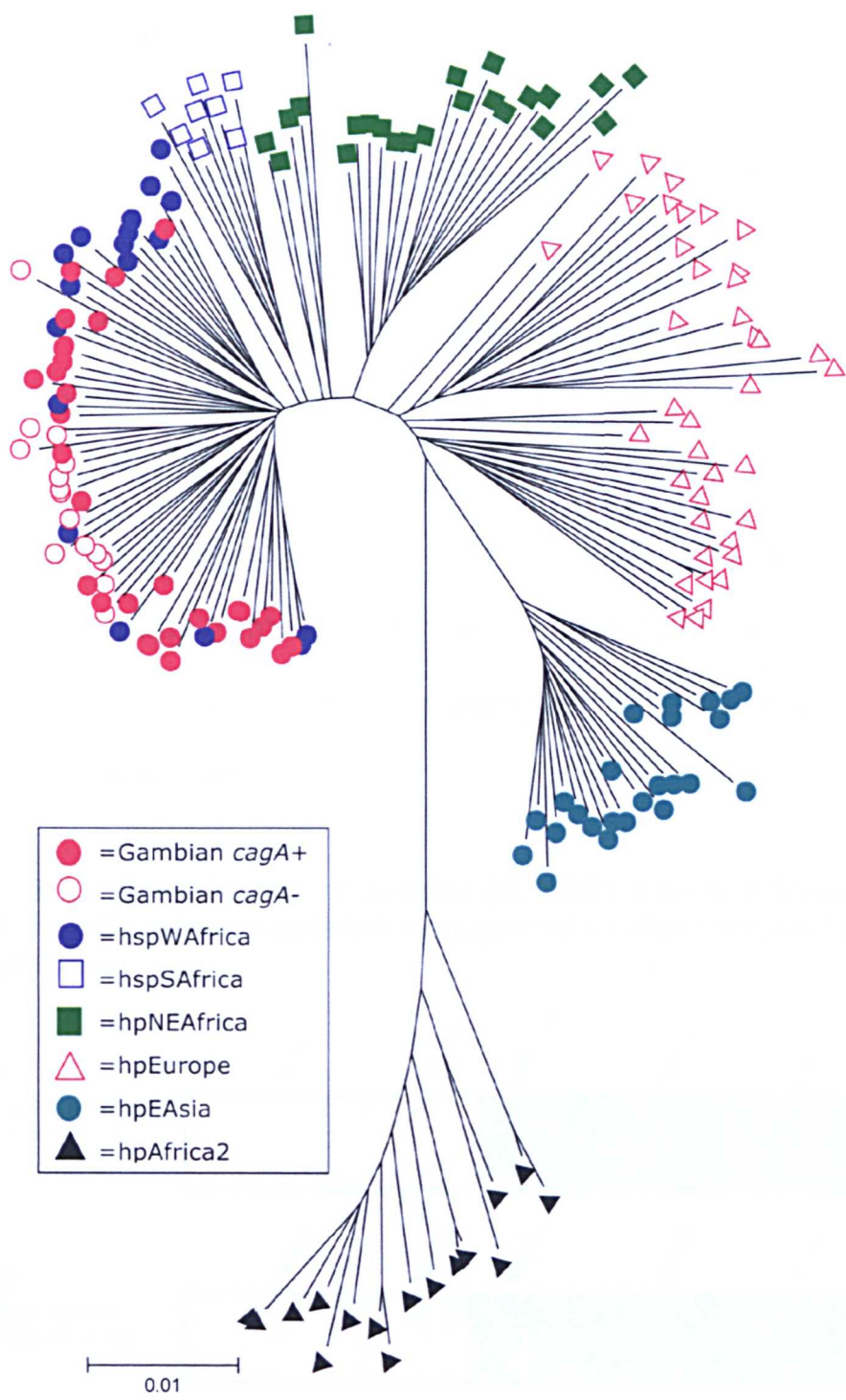
The Gambian *H. pylori* strains were closely related to but not intermingled with 14 strains from Burkina Faso, about 1600Km to the East of the Gambia, and also to the five available isolates from Senegal which borders The Gambia in all but its ocean side (figures 6.8 and 6.9).

Figure 6.8 Evolutionary relationship of global strains showing country of origin



The Neighbor Joining tree was calculated from concatenated sequences of seven housekeeping genes (3406 bp) from 128 isolates of *H. pylori* downloaded from mlst website (<http://pubmlst.org/Helicobacter>) plus 46 isolates from this study. The strains were colour-coded according to country of origins.

Figure 6.9 Evolutionary relationship of global strains showing population



The Neighbor Joining tree was calculated from concatenated sequences of seven housekeeping genes (3406 bp) from 128 isolates of *H. pylori* downloaded from mlst website (<http://pubmlst.org/Helicobacter>) plus 46 isolates from this study. The strains were colour-coded according to populations.

With the no-admixture model, almost all Gambian isolates formed a homogeneous group that belonged to hpAfrica1 (figure 6.10A) essentially with no traces of European or North African ancestry despite Gambia’s history of invasion and colonisation by peoples from these regions during the last millennium. With the admixture model, the proportion of other ancestral nucleotides in Gambian strains was lower than the hpAfrica1 group (hspWAFrica and hspSAfrica, figure 6.10B). Most Gambian strains were clearly distinct from (although related to) strains of the hspSAfrica and hpNEAfrica populations (figures 6.10A and 6.10B) and also to other hspWAFrica strains from North Africa (figure 6.10B). We only found one isolate with significant evidence of European ancestry in a 57 year old female (figure 6.10B).

Figure 6.10 No-admixture model (6.10A) and admixture model (6.10B) of Gambian isolates compared with previously assigned populations.

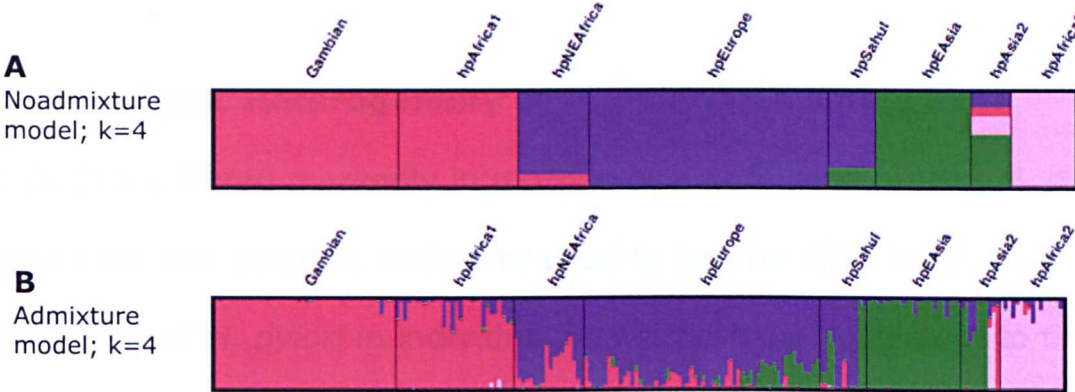
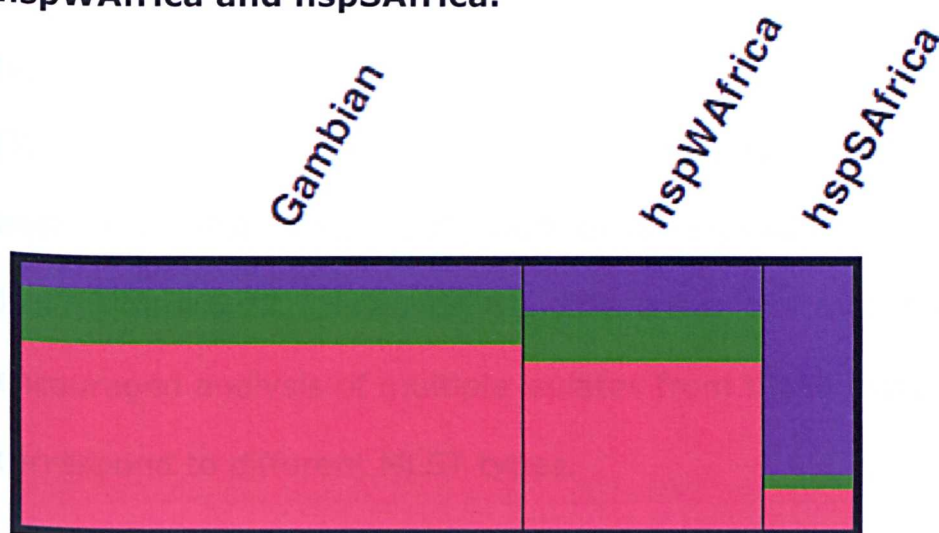


Figure 6.10A: Noadmixture model of seven populations and Gambian isolates. Each isolate is represented by a thin line that is colour coded according to the population assignment.
Figure 6.10B: Admixture model of seven assigned populations and Gambian isolates. Each isolate is represented by a thin line that indicates the estimated amount of ancestry from each of the seven ancestral populations.

Further STRUCTURE analysis of Gambian with hspWAfrica and hspSAfrican strains showed that with the admixture model, Gambian strains were closely related to hspWAfrica and less closely related to strains from more distant African regions (figure 6.11).

Figure 6.11 Admixture model of Gambian isolates compared with hspWAfrica and hspSAfrica.



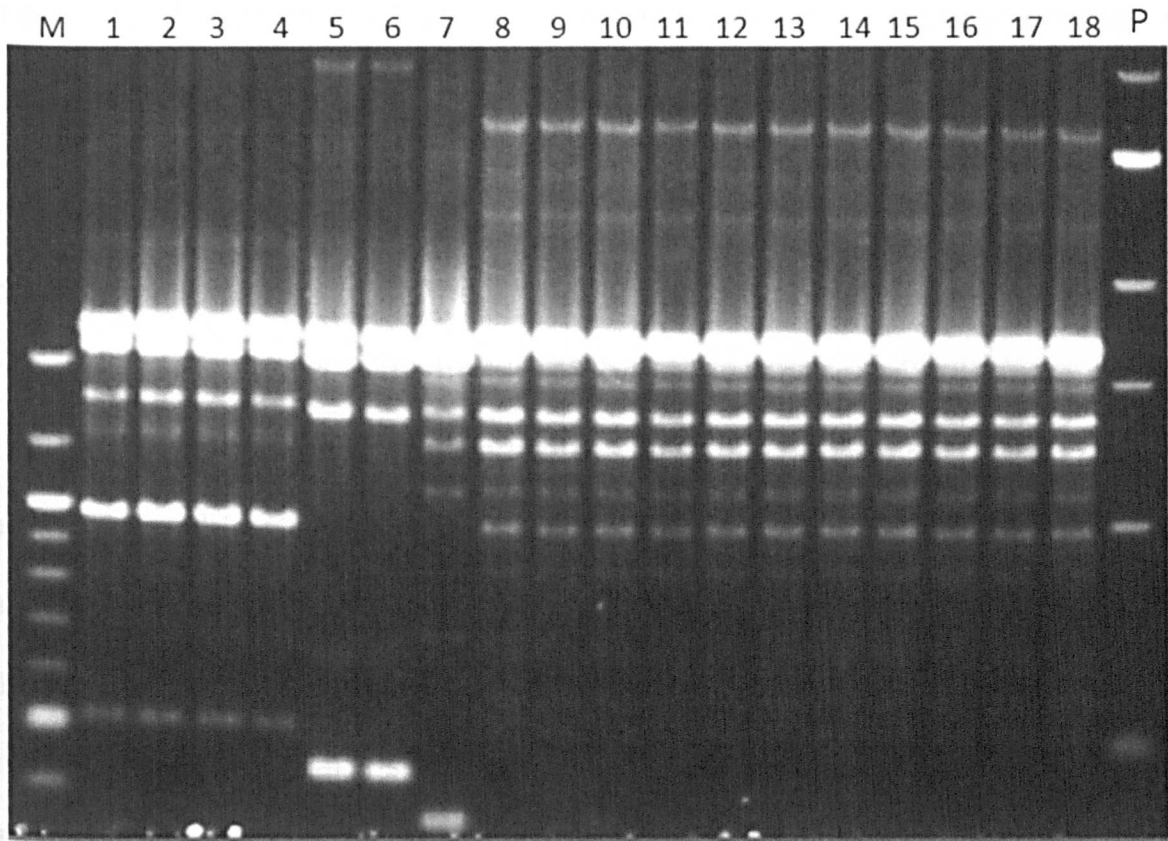
Admixture model of Gambian isolates compared with previously assigned populations (hspWAfrica and hspSAfrica). Each line represents an isolate colour coded according to the assigned populations and indicates the estimated amount of ancestry from each of the known ancestral populations.

6.4.6 Phenotypic Heterogeneity of *H. pylori* in a single host

Colonies that differed markedly in morphology were seen among *H. pylori* cultured from two persons, and were used to test for DNA level heterogeneity of *H. pylori* in individual hosts, perhaps equivalent to that seen previously in a European with a mixed *cagA*⁺ and *cagA*⁻ [186]. From one person (14 years) with normal gastroduodenal tract appearance by endoscopy, 7 colonies were analysed (4 antrum, 3 body). From the other person (72 years), also with normal gastroduodenal tract by endoscopy,

11 single colonies were sub-cultured and analysed (6 antrum and 5 body). Three different RAPD profiles and MLST types were identified among the seven isolates from the 14 year old, represented by MLST types 2040 from antrum, and 2047 and 2064 from body; figure 6.11) which were different in all seven gene loci tested. The four colonies from the antrum were identical to one another and different from the two MLST types found among three colonies from the body. In the second patient (72 years), all colonies 6 antrum and 5 body were identical to one another. Similar RAPD results were observed with both primers (1254 and 1283; figure 6.12). However, the different colony morphologies that first encouraged analysis of multiple isolates from these two patients did not correspond to different MLST types.

Figure 6.12 RAPD-PCR profiles (primer 1254) of strains isolated from two individuals.



M= molecular markers (100bp); P = molecular marker (1kb);
Lanes 1 – 7 = strains from 14 year old (1-4 from antrum, 5-7 from body);
lanes 8-18 from 72 year old (8-13) from antrum, (14-18) from body.

6.5 Discussion

Most detailed studies and analyses of *H. pylori* populations to date have used strains from non-African countries, despite the great importance of events in Africa for the emergence and evolution of humans, and of diverse infective agents, probably including *H. pylori*. Here we used MLST to analyse strains from The Gambia, the most detailed study to date of an *H. pylori* population from West Africa. These Gambian strains exhibited the high degree of nucleotide sequence diversity described in other

groups of isolates from defined geographic regions strains [181,303] with no obvious clustering of MLST types in particular age or disease groups.

We have shown that the strains of *H. pylori* found amongst Gambian residents are typical of hpAfrica1, and their inclusion within this population allowed us to define and describe the population with more precision. Comparison with the handful of sequences available from Burkina Faso, which is inland and to the East of The Gambia, suggests some geographic differentiation even within West Africa. This divergence could be due to isolation by geographic distance and/or linguistic or ethnic differences and/or human population history, each of which could foster random genetic drift and selection for locally adapted lineages.

STRUCTURE analysis indicated that the contribution of ancestral strains from other populations to strains circulating in The Gambia was <1%. Gambian strains were closely related to each other and form part of the hspWAfrica subpopulation of strains (figures 6.10). The high “purity” of Gambian *H. pylori* despite the country’s significant exposure to North Africans and Europeans throughout its history, contrasts interestingly with patterns found in strains from Amerindian and Mestizo Latin Americans [210]: many of these Latin American strains seem to be mosaic, with significant and complex European, African and/or Amerind ancestries [95,210].

Different *H. pylori* populations were also not observed in strains from neighbouring Senegal or from Burkina Faso. This contrasts with *H. pylori*

diverse populations found in rural South Africa [89]. A set of Northern Nigerian strains were hpNEAfrica [160]. Although not necessarily expected, it is not surprising as these strains were isolated from Borno state in Northern Nigeria, which is populated by the Kanuri people who were pastoral berbers with roots in Yemen, migrating and settling in North Africa during twelfth century. During the height of the trans-Saharan trade, the Kanuris moved south via Libya before settling in the Lake Chad region including Borno [304].

6.5.1 Identical MLST types

Four pairs of strains of identical MLST types were found in this study. One pair of was from consecutive unrelated patients, who had biopsies taken on the same day. However, we think that this occurrence is not likely to be due to cross contamination during endoscopy or sample processing in the laboratory as standard endoscopy procedures (SOP-CLS-001) were followed; using clean endoscopes sterilized with Cidex (Johnson and Johnson Co) and rinsed with clean water between cases, according to standard care at MRC Unit, The Gambia.

The other three pairs were from people who had their biopsies taken a week to two years apart and also processed in the laboratory on different dates. No strain pairs with identical MLSTs were from persons from the same village or with same family names. Further study will be needed to learn if the people carrying these matched strains had ever lived in the

same extended family compound, village or district, or had some other connection, vs. if such identical MLST types reflect some other factor such as The Gambia's small size and easy hospitality to strangers.

Given the lack of obvious connection between these paired strains, genome-wide analyses of their patterns of micro-sequence divergence vs. conservation could also be highly informative.

6.5.2 Heterogeneity of *H pylori* strains within one stomach

In two patients tested for possible heterogeneity, all colonies from one patient were identical by MLST whilst the other had three distinct MLST types consistent with other findings [89,101,186]. The sequence types of these three strains were different in all seven of the gene loci scored, thereby suggesting intriguing co-infection among unrelated strains [253,277].

6.6 Conclusion

This study indicated that Gambian *H. pylori* are not particularly clonal, in accord with patterns seen in other non-African populations. Since the MLST types of the strains obtained from young children were intermingled with those of adults, we suggest that there may not be any special strain type uniquely able to initiate infection in naive infant stomachs. We also note that our strains from the far Western part of Africa showed more genetic similarity with strains from Senegal and Burkina Faso than from elsewhere, reflecting again geographic partitioning of *H. pylori*. The

relative paucity of admixture of DNA sequences of European and North African origin in Gambian *H. pylori* strains in contrast to that seen in Latin American populations, despite the historical importance of foreigners in The Gambia suggests to us that hspWAfrica *H. pylori* strains might be as or more fit than their European and North African competitors. If correct this inference would have implications for *H. pylori* colonisation and disease in the African diaspora-among people with African ancestry minorities in the Americas and Europe, as well as zones of contact between northern and sub-Saharan peoples in the African continent. The strain collection and database generated in this study should be useful for further examination of issues such as strain virulence which normally would spread to colonise new areas with the African diaspora or host genetics with the colonising strain staying more or less in those of African descent.

Chapter 7 Antimicrobial susceptibility and resistance patterns among *Helicobacter pylori* strains from The Gambia, West Africa

7.1 Introduction

Helicobacter pylori chronically infects most people in developing countries [102,305], typically starting in infancy [19,102,103] and lasting for life. It also remains a significant pathogen in industrialized countries, infecting some 10-40% of adults in many societies. Chronic *H. pylori* infection is a major cause of gastric (stomach) and duodenal ulcers and gastric cancer [69,71,73]. It also increases the risk of infection by other gastrointestinal pathogens, iron deficiency anaemia, and infant malnutrition and growth faltering, especially among the very poor [141,251]. These latter conditions are of particular concern in The Gambia, a small developing country on the West Coast of Africa. Fortunately many *H. pylori*-associated illnesses can be prevented or cured by timely eradication of the bacterium, which typically entails one-two weeks treatment variously with metronidazole (Mtz), amoxicillin (Amo), clarithromycin (Cla), when affordable, and/or tetracycline (Tet), in combination with a proton pump inhibitor such as omeprazole, and/or bismuth where allowed by local regulations [215]. *H. pylori* transmission tends to be highly localized and preferentially intrafamilial [89,101] in industrialized societies, and often also between households in the local community in developing country settings [306]. Given relatively localized transmission, successful eradication from many members of a household or community might markedly diminish the risk of new infections, especially of newborns, and

thereby contribute importantly to public health.

Resistance to useful antimicrobials, especially Mtz and Cla, has been a major problem in some societies, even among people not previously treated for their *H. pylori* infections. Such resistance is generally attributable to inadvertent *H. pylori* exposure during treatment for other conditions [226]. Mtz itself is an innocuous pro-drug that is activated by chemical reduction to hydroxylamine type compounds, which are bactericidal to *H. pylori* [225]. In the strains studied to date, mostly from industrialized societies, a modest level of Mtz resistance (e.g. to 8 or 16µg Mtz/ml) was usually associated with inactivation of the gene *rdxA*, which encodes a non-essential oxygen-insensitive NADPH nitroreductase that chemically reduces Mtz in vitro [222]. Higher level resistance in *rdxA* mutant strains, e.g. to 32µg Mtz/ml and above, resulted from inactivation of *frxA*, a related but generally less strongly transcribed nitroreductase gene; yet higher level resistance can result from mutations in any of several additional genes that likely also affect intracellular redox potential [222-224]. The hydroxylamine-type derivatives of Mtz that RdxA protein generates are mutagenic, such that exposure to sub-lethal Mtz concentrations [225] induces as well as selects for mutations to Mtz resistance.

No commonly used anti-*H. pylori* drugs other than Mtz are known to require activation to render them bactericidal, nor to be so highly

mutagenic. Additionally, the several resistances to these other drugs identified to date involve specific mutational changes that alter the target's function. In particular, resistance to the related macrolides erythromycin (Ery) and clarithromycin (Cla), which are used in anti-*H. pylori* therapy, is usually achieved by point mutations at either of two adjacent sites in 23S rRNA [230,307] that diminish macrolide binding to the ribosome. Cla resistance seems to be rare in many societies, but common (more than one-fourth of strains) in others [216,308]. The observed prevalence probably reflects a combination of the very few rRNA sites in which sequence changes can confer resistance and are not too deleterious for the bacterium, a need to incorporate any resistance mutation in both 23S rRNA genes to achieve a resistance phenotype, and the intensity of macrolide use for other infections and thereby inadvertent exposure of resident *H. pylori* strains.

Tet resistance is much rarer than Mtz or Cla resistance [233,236], although several bona fide resistant strains have been identified and analyzed. In the best described case, modest resistance resulted from three contiguous changes in the Tet binding pocket in 16S rRNA (positions 965-967) [236,237]. Lower level resistance was achieved by mutation at one or two of these positions and/or by mutations in genes in other chromosomal locations that have not yet been identified, but are suspected to affect bacterial permeability or efflux [236]. Amo resistance is also very rare, but where found has been ascribed to mutation in a

penicillin-binding protein involved in cell wall metabolism [235,309-312].

The present study of drug susceptibility and resistance in Gambian *H. pylori* strains was motivated in part by considering that *H. pylori* is a genetically very diverse species, with different genotypes predominating in different well separated geographic regions, even in different parts of Africa [160]; and that most studies of drug susceptibility and resistance have focused on strains from Europe, The Americas, or Asia. As with many infection-related topics, there have been far fewer critical studies of antimicrobial resistance and susceptibility of *H. pylori* strains from Africa, especially those from West Africa – the ancestral home of most people of African ethnicity in The Americas. Given *H. pylori* transmission preferentially within families and local communities [89,101,306], West African strains may well have contributed to *H. pylori* gene pools in The Americas.

Bearing in mind *H. pylori*'s impact on public health worldwide, The Gambia included, and the distinctiveness of African strains, here we assessed the frequencies of resistance to Amo, Cla, Ery, Mtz and Tet in a set of 64 strains from Gambian citizens. We tested the importance of *rdxA* status for Mtz susceptibility and resistance by transformation and DNA sequence analysis, and interpreted amino acid sequence differences in RdxA protein in terms of its recently determined structure [313]. We also tested by DNA sequencing whether 16S rRNA gene mutations could be

responsible for the very few Tet resistant isolates found as minority components of mixed *H. pylori* populations from several patient biopsies.

7.3 Results

7.3.1 Metronidazole susceptibility and resistance

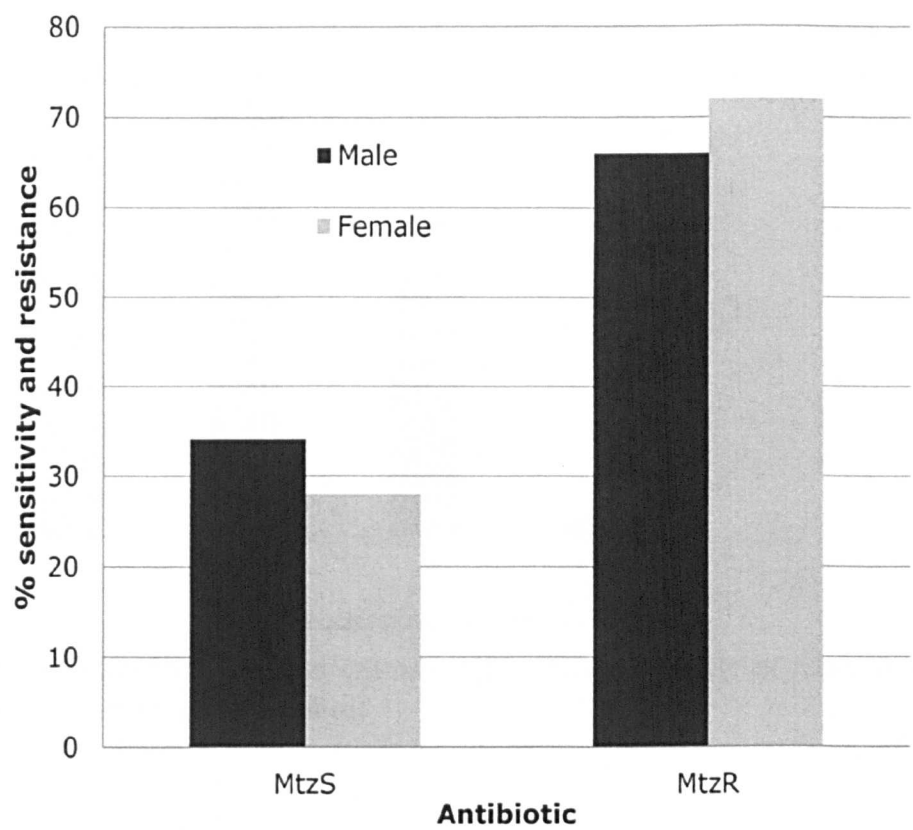
Of the 64 strains tested for Mtz susceptibility, 20 (31.2%) were sensitive (single cells unable to form colonies) on media with Mtz at 8µg/ml and 44 (68.8%) were resistant. All six strains from young children (18-31 months) were sensitive to this modest level of Mtz, whereas only 14 (24%) of 58 strains from adults were sensitive (P=0.0031; table 7.1).

Table 7.1 Minimum Inhibitory Concentration (MIC) for Mtz against *H. pylori* isolated from males vs females

		Range of MIC for Mtz (µg/ml)						Total
Age group	Sex	<8	8	16	32	64	128	
Adult	Male	8	2	8	12	1	0	31
	Female	6	1	5	14	1	0	27
Infant	Male	4	0	0	0	0	0	4
	Female	2	0	0	0	0	0	2
Total		20	3	13	26	2	0	64

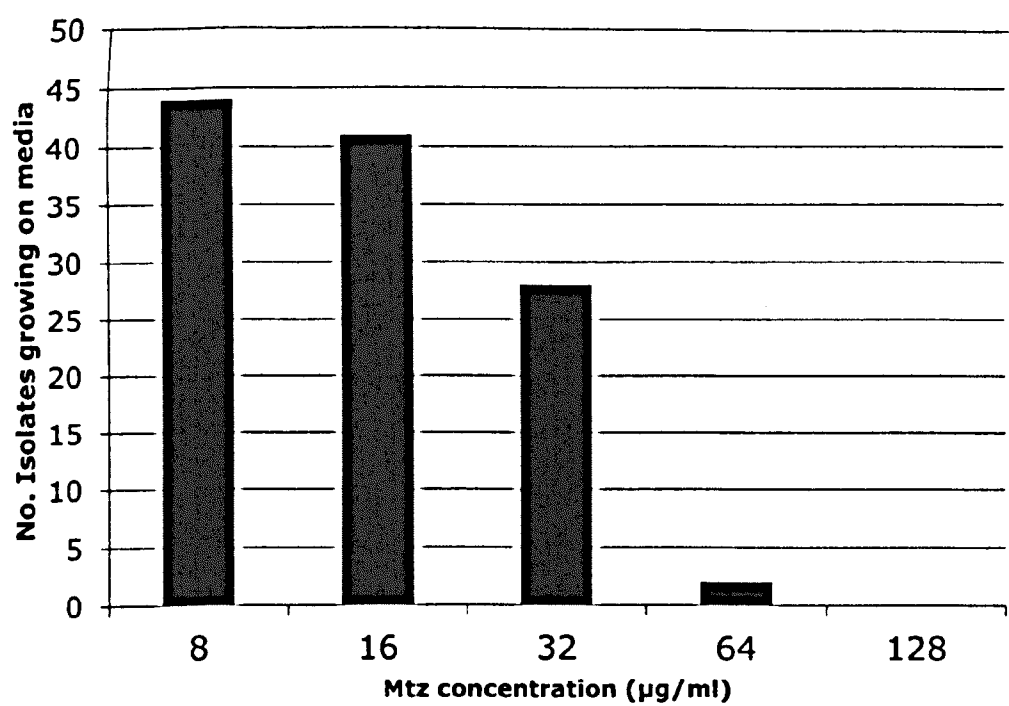
The prevalence of Mtz resistance in males vs. females was 66% vs. 72% respectively but the difference was not statistically different (P > 0.05; table 7.1 and figure 7.1).

Figure 7.1 Prevalence of antibiotic resistant isolates in males and females



All but three of the 44 strains that were resistant to 8µg Mtz/ml also grew well on medium with 16µg Mtz/ml. Additionally, 28 of these strains also grew on medium with 32µg of Mtz/ml; and two of the 28 grew on medium with 64µg Mtz/ml. None of our 44 strains grew on medium with 128µg Mtz/ml (figure 7.2).

Figure 7.2 Number of isolates growing on metronidazole medium



The numbers of isolates that formed colonies on different levels of metronidazole medium were determined and graphed.

In further tests of strains that did not grow with 8µg Mtz/ml, two of the six from infants (18-31 months), and two of fourteen representative strains from adults grew on medium with only 4µg of Mtz/ml.

7.3.1.1 *rdxA* (nitroreductase) gene analysis

A transformation test was used to learn if Mtz^S strains were distinct metabolically from most susceptible reference strains, in requiring more than just *rdxA*-inactivation to achieve Mtz resistance [222-224]. Eleven Mtz sensitive strains were transformed with genomic DNA from a derivative of strain 26695 whose *rdxA* gene had been replaced with a chloramphenicol-resistant (Cam^R) cassette (Δ *rdxA*-cat). Each of the 10-20

Cam^R transformant colonies tested from each of the 11 strain transformations grew well on agar with 8µg Mtz/ml. This outcome indicates that most or all Mtz^S Gambian *H. pylori* strains are just one mutational (*rdxA* inactivation) step away from becoming resistant. In a converse experiment, we tested if mutation in *rdxA* was important for the resistance of Mtz^R Gambian strains. This entailed transforming 12 representative Mtz^R strains with genomic DNA from an *H. pylori* strain containing a kanamycin-resistant (Kan^R) cassette inserted next to a functional *rdxA* gene. We expected that a fraction of Kan^R transformants would acquire the donor strain's *rdxA*⁺ (functional) allele [314], even though most might retain the recipient *rdxA* mutant allele because *H. pylori* transformation tends to involve mostly short DNA fragments [315]. At least two of the 20-30 Kan^R transformants scored from each of 12 Mtz^R recipient strains were found to be Mtz^S on agar with 8µg Mtz/ml, even though most Kan^R transformants remained Mtz^R. We infer that these few Kan^R Mtz^S transformants had gained the donor's functional *rdxA* allele, and thereby conclude that *rdxA* inactivation is needed for most or all Mtz^S Gambian *H. pylori* strains if they are to become Mtz^R.

7.3.1.2 Sequence comparison of *rdxA* from Mtz^R and Mtz^S strains.

The *rdxA* gene was PCR amplified and sequenced from 33 Mtz^R (MIC range 8-32µg/ml) and 18 Mtz^S Gambian strains (MIC <8µg/ml). Average *rdxA* sequence diversities were 3.6 % in Mtz^R and 3.4% Mtz^S strains (overall, 3.5%), which is within the range of diversities among Gambian

strain housekeeping genes (range, 1.2 – 4.6% mean, 2.9%), and whose protein products also act internally in these Gambian strains (observation made during experimental work for chapter 6).

Of the 33 Mtz^R strains characterized, 15 (45.5%) contained nonsense (translation stop) codons within the *rdxA* orf, including 13 of the 19 resistant to 32 µg Mtz/ml; in contrast only two of the 14 isolates with lower level resistance (8-16µg/ml) contained nonsense mutations in *rdxA* (p=0.004, table 7.2).

Table 7.2 *rdxA* nonsense and frameshift mutations ^a

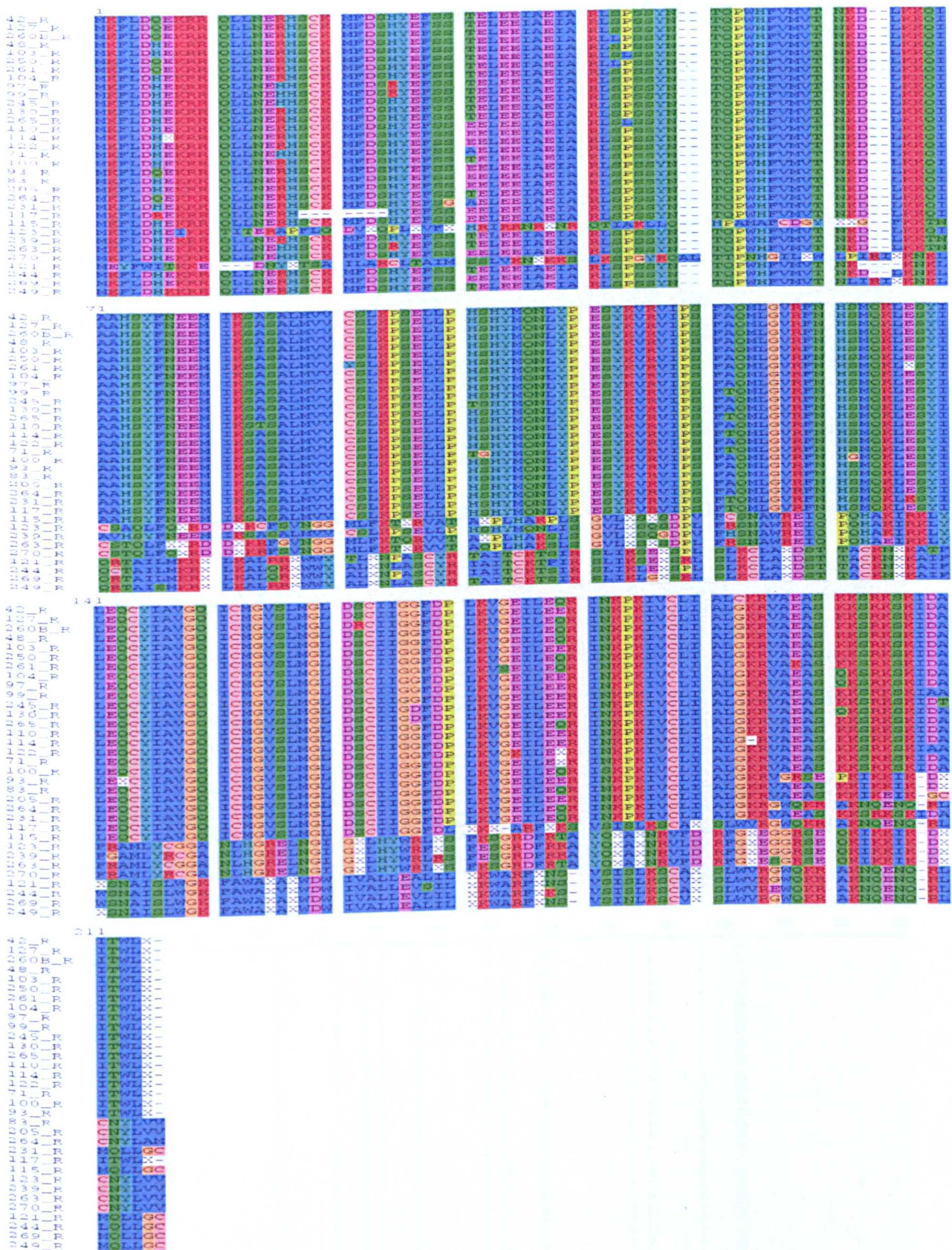
Strain	µg/ml	Frame shift	Mutation description (base position)	Mutation (codon #, base position) ^b
Mtz^R				
71R	32	0	^c Substitution (523) C or G -> T	Stop codon (175, 523)
83R	32	+1	Insertion (576)	Stop codon (205, 613)
93R	32	0	Substitution (415) C -> T	Stop codon (139, 415)
100R	32	0	^c Substitution (523) C or G -> T	Stop codon (175, 523)
114R	32	0	Substitution (19) G -> T	Stop codon (7, 19)
115R	32	-1	Deletion (496)	Stop codon (167, 499)
121R	16	+2	Insertion (6,7)	Stop codon (14, 40)
123R	32	+1	Insertion (23)	Stop codon (23, 67)
205R	32	+1	Insertion (595)	Stop codon (205, 613)
239R	8	+1	Insertion (313)	Stop codon (110, 328)
244R	32	-7	Deletion x7 (179)	Stop codon (74, 220)
249R	32	+5	Insertion x5 (178)	Stop codon (64, 190)
263R	32	+1	Insertion (193)	Stop codon (73, 217)
269R	32	-1	Deletion (191)	Stop codon (76, 226)
270R	32	+1	Insertion (193)	Stop codon (74, 220)
Mtz^S				
JS114S	<8	+4	Insertion x4 (300)	Stop codon (111, 331)
JS124S	<8	+4	Insertion x4 (300)	Stop codon (111, 331) frameshift +4
101S	<8	-1	Deletion (86)	Stop codon (33, 97)

^a Not listed in this table are other Mtz^R strains that contained a 3 nucleotide deletion at codon 191, a 21 nucleotide (7 codon) deletion starting at codon 18, or the many missense mutations that resulted in amino acid differences in the encoded protein relative to *rdxA* of reference strain 26695. These amino acid replacements are shown in figure 7.3.

^b Complete RdxA protein is 210 amino acids long

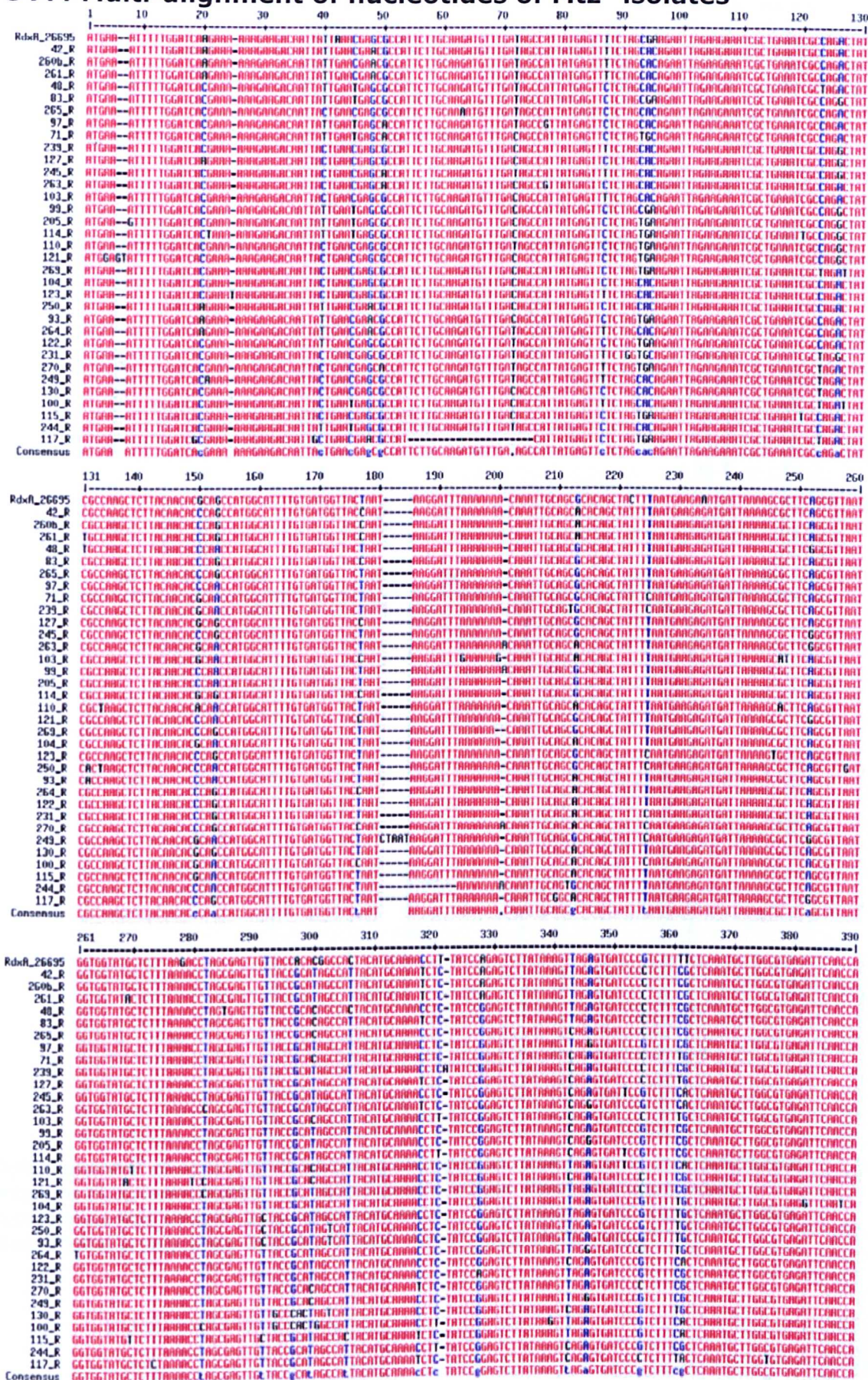
^c 17 isolates have a "C" and 14 "G" at position 523 (figure 7.4)

Figure 7.3 Amino acid sequences of metronidazole resistant isolates showing “stop codons” (X)



Multialignment of amino acids showing changes in *rdxA* gene of metronidazole resistant *H. pylori* isolates. *H. pylori* 26995 whose RdxA structure was determined was chosen as a reference strain. Multi-alignment was performed using Sea View Version 4.

Figure 7.4 Multi-alignment of nucleotides of Mtz^R isolates



This difference in distribution is in accord with nonsense mutations causing protein truncation and thus, complete loss of RdxA function. Some missense mutations diminish but do not entirely eliminate an encoded protein's activity, and thus would confer only leaky phenotypes (lower level Mtz resistance in the case of *rdxA*), whereas many others would be well tolerated and have little if any effect on activity of the encoded protein.

Three of 33 Mtz^R (9.0%) strains had insertions of one or two nucleotide and thereby *rdxA* frameshift mutations, which would result in new amino acid sequences distal to the mutant site and thereby loss of *rdxA* function. In addition, two strains contained in-frame deletions of three and 21 nucleotides, which do not cause changes in RdxA protein sequences distal to the mutant sites (table 7.3).

Table 7.3 Identification of proteins with frame shifting mutations or with large in frame deletions of Mtz^R strains (excluding those already detected as non functional in table 7.2)

Lab No	MIC µg/ml	Mutation description	Mutational effect
99R	16	1 nucleotide insertion at nucleotide 201	Loss of function not reflected in translated sequence
264R	16	1 nucleotide insertion at nucleotide 607	Massive mutation from residue 199. Probable loss of FMN binding capacity
231R	8	2 nucleotides insertion at nucleotide 586	Massive mutation from residue 192. Loss of FMN binding capacity
122R	32	3 nucleotide deletion at nucleotide 580 (in frame)	Massive structural mutation due to loss of residue 191. Loss of FMN binding capacity
117R	16	large in frame deletion (1 nucleotide, 7 codon deletion)	Protein residues 18-24 missing. Not compatible with proper folding

Thirteen of 33 (39%) Mtz^R strains had neither translation stop nor indel mutations in *rdxA*, but their *rdxA* alleles differed from those in Mtz^S strains by numerous substitutions (table 7.4).

Table 7.4 Distribution of probable loss of function mutations in the following variants

Mtz Resistant isolates													Mtz Susceptible isolates														
42 R	260 R	261 R	48 R	265 R	97 R	110 R	127 R	245 R	103 R	104 R	130 R	250 R	js 106 S	80 S	33 S	119 S	125 S	128 S	131 S	260 S	268 S	js 117 S	js 123 S	105 S	js 136 S	50 S	252 S
D59N	D59N	S43L	Q6H	Q6H	Q6H	Q6H	D59N	Q6H	Q6H	Q6H	Q6H	P44L	Q6H	Q6H	Q6H	Q6H	Q6H	Q6H	Q6H	Q6H	Q6H	Q6H	D59N	D59N	Q6H	D59N	
R90K	R90K	D59N	S43L	D59N	R16H	T31E	R90K	R16H	D59N	D59N	D59N	D59N	Q11K	D59N	S30G	D59N	D59N	R16H	T31E	T31E	T31E	S30G	A67V	R90K	D59N	R90K	
G98S	G98S	C87Y	D59N	R90K	H25R	P44L	G98S	D59N	A80I	R90K	R90K	R90K	D59N	R90K	D59N	R90K	R90K	D59N	D59N	D59N	D59N	T31A	R90K	R90K	G98S	R90K	G98S
R131K	R131K	R90K	R90K	G98S	D59N	D59N	R131K	R90K	R90K	G98S	H97T	G98S	A68V	G98S	G98S	G98S	G98S	R90K	R90K	R90K	R90K	D59N	G98S	R90K	G98S	R131K	R131K
V172I	S158R	G98S	G98S	R131K	R90K	A80T	V172I	G98S	G98S	R131K	G98S	R131K	R90K	R131K	R131K	R131K	A118T	R131K	R131K	R131K	R131K	R90K	R131K	V172I	R131K	R131K	V172I
E175Q	V172I	R131K	R131K	G163D	G98S	R90K	A183V	A118T	R131K	E133X	R131K	V172I	G98S	V172I	E175Q	V172I	V172I	R131K	A118T	V172I	V172I	E175Q	V172I	E175Q	A183V	E175Q	Q197K
A183V	A183V	V172I	V172I	V172I	R131K	G98S	Q197K	R131K	V172I	G170S	G163D	A183V	R131K	R131K	A183V	A183V	A183V	A183V	V172I	A183V	A183V	R131K	A183V	A183V	Q197K	A183V	V204I
G189C	Q197K	A183V	E175Q	A183V	V172I	A118T	V204I	V172I	E175Q	V172I	V172I	Q197K	V172I	V172I	A183V	Q197K	Q197K	Q197R	A183V	Q197K	Q197K	V172I	Q197K	Q197K	V204I	Q197K	V204I
Q197K	V204I	Q197K	A183V	V204I	A183V	R131K		A183V	A183V	E175Q	A183V	V204I	A183V	A183V	V204I	V204I	V204I	V204I	Q197R	A183V	Q197K	V172I	Q197K	Q197K	V204I	Q197K	
V204I		V204I	Q197K		V204I	V172I		Q197K	Q197K	A183V	Q197K		Q197K	Q197K	V204I	V204I	V204I	V204I	Q197K	V204I	V204I	E175Q	V204I	V204I	V204I		
			V204I			E175Q		V204I	V204I	E194K	V204I		V204I						V204I			A183V					
						A183V		D205A		Q197K	A206T											Q197K					
						Q197K				V204I												V204I					
						V204I																					

The mutations tentatively classified as loss of function of 13 Mtz^R and 15 Mtz^S isolates are marked in yellow, and those present in the segment not seen in the x-ray structure are shown in blue

Table 7.5 Mutations found in only Mtz^R or Mtz^S isolates

Mutations only in Mtz^R isolates	Number of occurrences		Mutations only in Mtz^S isolates	Number of occurrences
H25R	1		S30G	2
S43L	2		T31A	1
P44L	2		A67V	2
A80I	1		A68V	1
A80T	1		Q197R	1
C87Y	1			
H97T	1			
E133K	1			
E194K	1			
S158R	1			
G163D	2			
G170S	1			
G189C	1			
D205A	1			
A206T	1			

The basis of Mtz resistance in two strains (127R and 104R, MIC 32µg/ml) is not clear from RdxA structure considerations. Their 210 residue RdxA proteins differ at many positions (6, 133, 170 and 194; figure 7.5) from the RdxA protein whose structure was determined (Hp0954 of reference strain 26695).

Figure 7.5 Multi-alignment of strains 104R and 127R



Multialignment of amino acids showing changes in *rdxA* gene of 104 and 127 metronidazole resistant *H. pylori* isolates. *H. pylori* 26995 whose RdxA structure was determined was chosen as a reference strain. Multi-alignment was performed using Multiple sequence alignment with hierarchical clustering [316].

However, all the differences present in strain 127R (D59N, R90K, G98S, R131K, V172I, A183V, Q197K, V204I) and most (Q6H, D59N, R90K, G98S, R131K, E133K, G170S, V172I, E175Q, A183V, E194K, Q197K, V204I) in 104R were also present in many other resistant and susceptible strains and thus may not perturb function (table 7.4). We interpret that most of those differences simply reflected neutral mutations in accord with *H. pylori*'s great genetic diversity. None of the three mutations that are specific to 104R (E133K, G170S, and E194K) is expected to affect RdxA function because they appeared on the protein surface far from the FMN binding site. Thus, why strains 127R and 104R were resistant is not obvious: possibly their particular combinations of changes in RdxA diminished function (conferred resistance); or these strains might have polar mutations in the upstream DNA that was not sequenced.

Among the 18 *rdxA* sequences from Mtz^S strains, three (17%) had internal stop codons (table 7.2) and three others had point mutations that also might lead to *rdxA* inactivation: R16H (in one strain) because it should decrease RdxA affinity for FMN's negatively charged phosphate; and A67V (in two strains), because it entails replacement of small alanine by bulky valine in the protein core, although direct tests will be needed to learn how severely this replacement affects protein stability and function. If these mutations do indeed cause *rdxA* inactivation, the Mtz^S phenotypes might stem from high-level *frxA* expression [222,224]; the possibility of nonsense suppressor mutant tRNAs in certain strains also merits consideration. The nonsense mutations between Mtz^S and Mtz^R strains to 32µg Mtz/ml was significant (p=0.0025, table 7.2), however, overall, the nonsense mutations between Mtz^S and Mtz^R strains was not (P=0.065, table 7.2).

Fifteen substitutions were found only in Mtz^R isolates (H25R, S43L, P44L, A80I, A80T, C87Y, H97T, E133K, S158R, G163D, G170S, G189C, E194K D205A and A206T; table 7.5), which suggests that some of them might decrease RdxA function. Conversely, five were only found in Mtz^S isolates (S30G, T31A, A67V, A68V and Q197R; table 7.5) and thus might be neutral (of these five, only A67V is suspected of decreasing RdxA function, as noted above).

7.3.2 Susceptibility of Gambian strains to other antibiotics.

All 64 of our Gambian *H. pylori* cultures grown directly from gastric biopsies were found to be highly sensitive to the closely related macrolides Cla and Ery. All cultures were also Tet and Amo sensitive (table 7.6), although one and four of them contained rare Amo and Tet resistant cells able to grow on medium with 2µg of Amo or Tet/ml, respectively (frequencies of 10^{-3} - 10^{-4}).

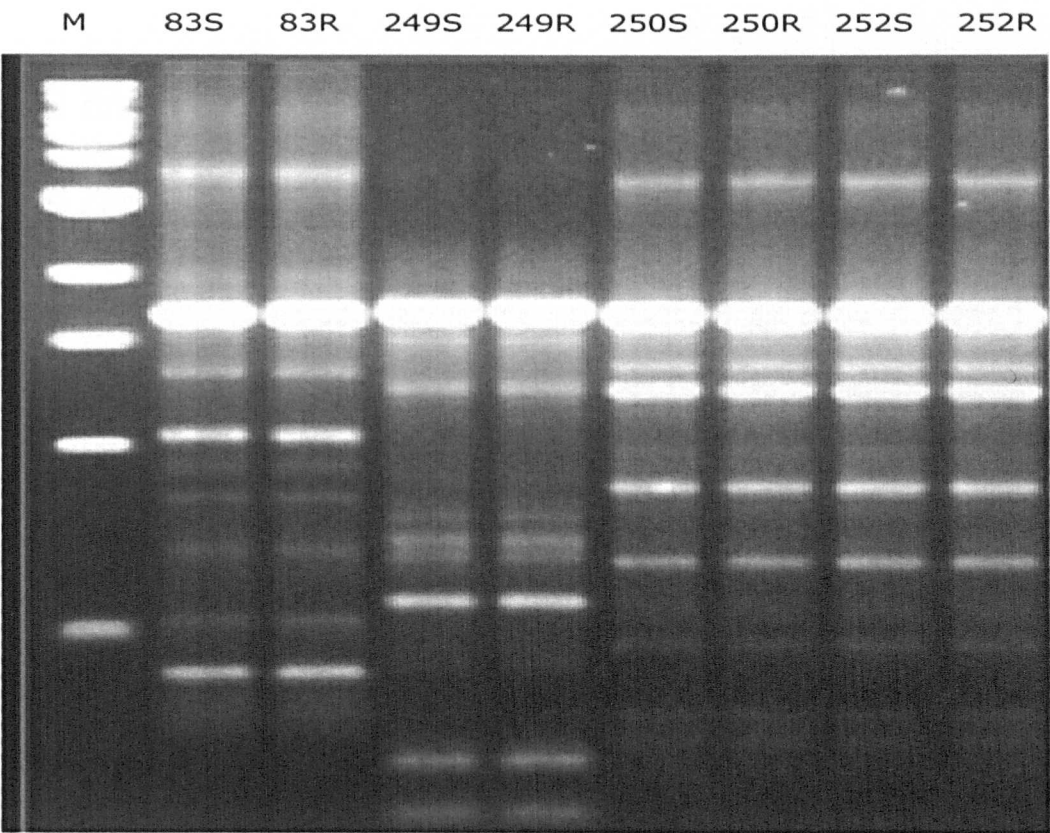
Table 7.6 Susceptibility of Gambian *H. pylori* strains to amoxicillin, clarithromycin, erythromycin and tetracycline

MIC (µg/ml)	Amo	Cla	Ery	Tet
<2	64	64	64	64
2	0	0	0	0
8*	-	-	-	-
16*	-	-	-	-
32*	-	-	-	-
64*	-	-	-	-
128*	-	-	-	-

* Strains sensitive at 2µg/ml were not tested at higher concentrations (8-128µg/ml).

Further tests of one Tet^R colony from each of these unusual Tet^R subclone-containing cultures showed that their MICs ranged from 2-4µg Tet/ml and that each was indistinguishable by RAPD-DNA fingerprinting from the predominant Tet^S strains from the same biopsy (figure 7.6).

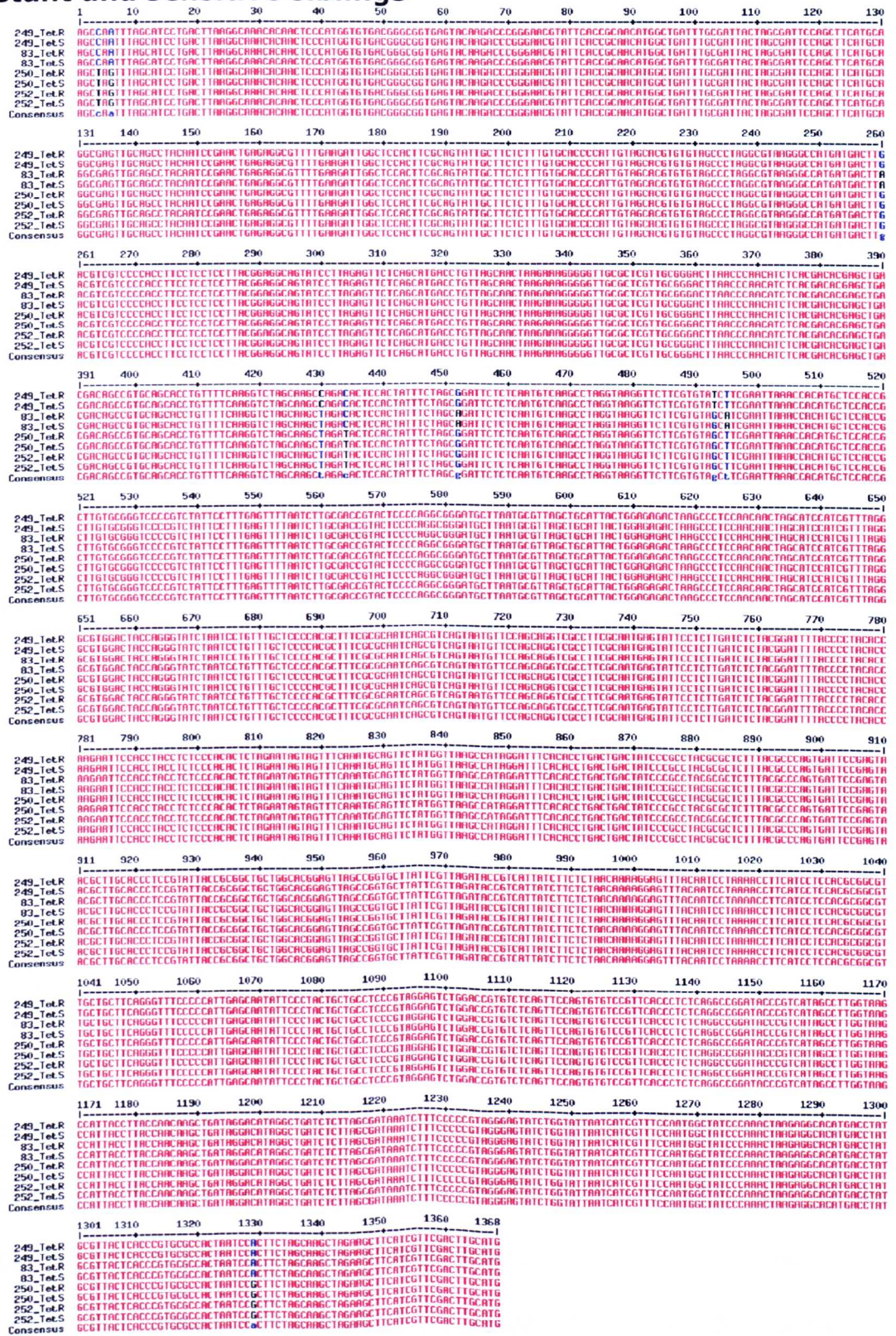
Figure 7.6 RAPD profiles of four strains with TetR colonies



Lane M is a kb DNA ladder (Biolabs, UK), "S" = Tet sensitive cells; "R" = Tet resistant cells

These four sets of strains were not closely related to one another, as expected, since they came from different persons. We found that PCR-amplified 16S rDNA of each Tet^R strain was identical in sequence to that of its Tet^S sibling from the same biopsy (figure 7.7). We conclude that in each of these four cases, Tet resistance is due to mutation in a gene distinct from that for 16S rRNA.

Figure 7.7 Multiple sequence alignment of tetracycline resistant and sensitive siblings



7.4 Discussion

H. pylori infection contributes importantly to several human diseases in both developing and industrialized countries, and directly impacts on health care systems worldwide. Its public health impact is of particular concern in developing countries because the prevalence of infection is so very high [102,305].

Here we scored susceptibility and resistance to clinically relevant anti-*H. pylori* agents using a test of efficiency of colony formation by single cells. This test is especially useful for scoring susceptibility to Mtz because Mtz can be mutagenic as well as bactericidal; it both induces and selects for resistance mutations [222,224,225]. We found that more than two-thirds of Gambian *H. pylori* strains were Mtz resistant. This high prevalence can be explained by the relative low-cost and easy availability of Mtz in The Gambia, as is typical of developing countries worldwide. Our results are in accord with other reports of many Mtz resistant strains elsewhere in Africa [109,169,221,228,317,318], India and Latin America [21,319]. Typically somewhat less than half of *H. pylori* strains from Europe and North America have been found to be Mtz^R [216], likely reflecting the tighter control of Mtz usage in industrialized than in developing countries. Our observation that all six strains from infants (18-31

months of age) were sensitive to 8µg of Mtz/ml, in contrast to 24% of those from older people (≥ 14 years) ($P = 0.003$) also merits further examination, especially on the possibility that *rdxA* function might contribute to fitness during establishment of infection.

Prior studies with other sets of strains had shown that Mtz resistance typically involves inactivation of *rdxA*, which encodes a nitroreductase that converts Mtz from prodrug to bactericidal agent by chemical reduction. However, *H. pylori* strains also contain a related gene, *frxA*, which also confers Mtz susceptibility if highly expressed, independent of *rdxA* status [222,229]. DNA transformation studies performed in this study indicated that each of 11 Mtz^S Gambian strains tested required only *rdxA* inactivation to gain a Mtz^R phenotype, and conversely, that resistance involved *rdxA* inactivation in each of the 12 Mtz^R strains tested.

Sequence analysis of *rdxA* genes from study strains identified loss of function mutations that should cause Mtz resistance similar to those found in previous studies [222,224,320]. In particular, *rdxA* nonsense (stop codon) mutations were more common in strains with moderate level resistance (32µg/ml) than in strains with lower level resistance (8-16µg/ml), in agreement with other findings [222,224,320].

However, three of 18 Mtz^S strains had *rdxA*-null nonsense mutations and three others had point mutations that also possibly might result in inactive RdxA proteins. These observations are consistent with other reports [222,224,321,322] that *rdxA* inactivation need not always lead to Mtz resistance, and can be explained by postulating higher level expression of the related *frxA* nitroreductase gene [222,224].

We also found that most Mtz^R strains had mutations scattered across the *rdxA* gene that are not likely to contribute to their resistant phenotype. This is in accord with *H. pylori*'s well known great sequence diversity, here seen in the *rdxA* gene.

Increasingly frequent Cla resistance, up to one-fourth or more of *H. pylori* strains has been reported in Europe and North America [216,308]. High prevalence, where encountered, has been attributed to use of macrolides to treat respiratory infections [216,323-325]. In The Gambia, Ery is now routinely used to treat lower respiratory tract and skin infections. However, there was no evidence of resistance to Cla or Ery in the 64 *H. pylori* we tested in accord with results reported in a nearby country (Senegal) where only 1% of isolates were resistant to Cla [317].

Tet and Amo are also much used. Tet, especially, is cheap and readily available in local drug stores in The Gambia, often without prescription. However, its easy availability did not result in much resistance in Gambian *H. pylori* strains: Tet^R strains were recovered from pooled cultures from only four patients, and then only as very rare cells in the population. This outcome is in accord with the rarity of Tet resistance in *H. pylori* from other parts of the world [216,317,326]. None of the four Tet^R strains had mutational changes in the several allowed positions in the 16s rRNA Tet binding pocket that can result in modest Tet resistance [236,237]. Thus, by default, their resistances are likely to stem from mutations in other loci – a class of mutations previously interpreted as more easily achieved, but also likely to diminish *H. pylori* fitness [236]. We propose that the low probability of mutation at just a few specific rRNA sites and low fitness conferred by Tet resistance mutations in other genes explains the rarity of Tet^R *H. pylori*, despite considerable exposure, in The Gambia.

Resistance to Amo was also very rare in Gambian strains, much as has been reported for other geographic regions [216,326].

7.5 Conclusion

The increased prevalence of resistance to antibiotics used against *H. pylori* is of great concern especially in developing countries where the

costs of even the least expensive first line drugs are a burden to average citizens. This contributes to the urgency of monitoring antibiotic resistant strain frequencies to help clinicians effectively manage patients and their antibiotic regimens, and effectively deal with treatment failure. The rich repertoire of *rdxA* mutations found in our many Gambian *H. pylori* strains should be useful for future studies of RdxA structure and function, of how RdxA and its FrxA homolog make *H. pylori* susceptible to prodrugs such as Mtz, and the roles of these two nitroreductase enzymes in *H. pylori*'s central metabolic networks.

Chapter 8 Discussion of hypotheses, aims and objectives

The primary aims of this study which was to characterise and evaluate the putative virulent *H. pylori* genotypes in The Gambia in relation to clinical outcome have been fulfilled.

8.1 Putative virulent factors such as *cagA* and *vacA* influence disease outcome in The Gambia.

The association of *H. pylori* virulent genes such as *cagA* and *vacA* and gastric pathologies (gastric cancer, duodenal and gastric ulcers) have been well established in some studies [268-270]. However, several studies from different world regions have not detected such an association [21,79,80,93,268,327-329], an outcome suggesting the possibility of other virulence-modulating factors.

In The Gambia, virulence genes were indeed common similar to results in Western countries where gastric cancer is high [330]. The *cagA* oncogene and the *vacAs1m1* toxigenic alleles are often linked to severe gastric pathologies. In this study, it was found that *cagA* was significantly associated with gastro-duodenal disease but *vacA* was not. Therefore, this hypothesis was accepted only in respect to *cagA*.

8.2 Mixed colonization with different genotypes influences the development of gastroduodenal disease.

Most important of our novel finding was that the co-existence of *cagA*⁺ and *cagA*⁻ strains was significantly more common in patients with NUD than those with severe gastric diseases; however there was no association between mixed *vacA* alleles and gastroduodenal disease.

The study indicated that the coexistence of both *cagA*⁺ and *cagA*⁻ strains in a single host was protective against the development of gastroduodenal disease. Therefore, this hypothesis was accepted in respect to *cagA* status.

8.3 Children and adults in the Gambia are colonized by different strains of *H. pylori*, which therefore circulate amongst different age groups

The transition from childhood colonization to adult disease state is affected by multiple factors including evolution of genotypes of colonizing *H. pylori* strains themselves, driven in part by the host response to infection. The chronically infected adults are likely to have reached a "steady state" of adaptation between host and pathogen after a period of many years. Phylogenetic analyses of strains found that the MLST types found in children who have been recently colonised completely intermingled with MLST types of adults from the

same population suggesting that strains from both groups of study subjects were similar in conserved genes. Children and adult strains had both genotypic and phenotypic differences in relation to virulence genes and antibiotic susceptibility to Mtz respectively. Young children significantly carried less virulent strains than any of the adult groups and we also found that all children strains were sensitive to Mtz. The difference in Mtz sensitivity between adults and children isolates is an indication that Mtz resistance might have developed from intensity of use over time for the treatment of other infections (parasitic and vaginosis) which are common in our environment. During the course of this thesis, we have seen no evidence that different strains circulate in different age groups, but evidence to suggest that strains may evolve into new genotypes within individual host. Therefore, this hypothesis was not accepted.

8.4 Gambian isolates of *H. pylori* form a distinct phylogenetic family within the grouping of African derived strains.

The population structure of *H. pylori* from West Africa has previously been barely determined, albeit a few strains in the MLST data base despite the high levels of prevalence. This data set presented in this thesis reported the largest sample to date from West Africa determined by MLST. MLST of seven housekeeping genes showed that

H. pylori followed human migration out of Africa and identified seven *H. pylori* populations. We found the strains from this previously unstudied far West African population do form a distinct phylogenetic family within the grouping of African derived strains but show genetic similarity with hpAfrica1 particularly hspWAfrica which shows their possible common genetic origin and therefore this hypothesis was accepted.

8.5 Study objectives

8.5.1 Genetic relatedness and phylogenetic of isolates in a single stomach

In a European study, differences in colony morphology reflected genotypic characteristics in relation to *cagA*⁺ and *cagA*⁻ infection [186].

In this study, when we looked at different looking colonies from two individual stomachs, all colonies from one individual were identical by RAPD and MLST in accord in what was observed in Colombia [117].

The other individual had three distinct RAPD profiles and also three distinct MLST types consistent with other findings [89,101,186]. The sequence types of these three strains were different in all gene loci showing that one can have different strains of *H. pylori* colonising one or more sites of an individual stomach. Further, in the subject with multiple MLST types, it was believed to be a case of re-exposure to novel strains colonizing the individual rather than recombination of the

H. pylori. This shows that true mixed infections may have been due to repeat colonisation events and not to recombination, at least in this subject.

8.5.2 Prevalence of antibiotic susceptibility and resistance to amoxicillin, clarithromycin, metronidazole, erythromycin and tetracycline

The treatment of *H. pylori* and its eradication continue to be a challenge especially in developing countries despite being sensitive to many antibiotics [29,216,226,323]. The factors associated with the cure of *H. pylori* includes increase in resistance to commonly used drugs, high rates of re-infections [226] and poor compliance [219]. Studies in India and Peru have reported 73% and 43% [226] recurrence of *H. pylori* infection within the first year respectively in patients whose initial infection was successfully eradicated.

Several regimes have been evaluated and currently used for treating *H. pylori* infections but the optimal therapy has not yet being defined, more so in developing countries where this is dictated by availability and affordability. The choice of antibiotics for the treatment of diseases is usually driven by antibiotic susceptibility tests but this is rarely done in *H. pylori* infections. With the increase in antibiotic resistance, this may contribute to treatment failures.

In this study, we found that all Gambian *H. pylori* cultures were highly sensitive to Cla, Ery, Amo & Tet but resistance to Mtz was high and were only seen in adults especially in females. All strains from young children were sensitive to Mtz. We also found *rdxA*, an established locus that harbours mutation in many Mtz resistant isolates was responsible for most of the resistance in Gambian *H. pylori* isolates in accord with other findings.

In The Gambia, erythromycin routinely used to treat ALRI especially in children and more recently clarithromycin in some clinics is a cause for concern as current treatment regimes for *H. pylori* are often beyond the reach of most Gambians.

8.5.3 The usefulness of PCR in the diagnosis of *H. pylori* in The Gambia.

Presently, there is no universally accepted "gold standard" for the detection of *H. pylori*. Culture based techniques for bacteria are generally cost effective and relatively easy to perform and reproducible for most clinically relevant bacteria. However, the culture and isolation of *H. pylori*, a fastidious organism, is both challenging and technically difficult to perform. Gastric biopsies are often contaminated with gut and upper respiratory tract microbiota and so it requires a selective medium with various antibiotics and an antifungal [253] for its

successful isolation in the laboratory. Further, *H. pylori* is a micro-aerophile and requires micro-aerobic gaseous environment provided by either Gaspaks or micro-aerobic incubators. All these requirements make the culture and isolation of *H. pylori* expensive and technically challenging especially in resource poor countries.

Thus, various DNA based methods have been evaluated to determine an optimum method for the detection of *H. pylori* in biological specimens. The use of PCR was found to be slightly superior in our hands with a higher diagnostic suitability, faster and technically easier to perform.

However culturing *H. pylori* is still important for antibiotic susceptibility tests that could guide therapy especially in cases of persistent infection where the isolation of the organism is important [331,332]. The choice therefore of test for the detection of *H. pylori* from clinical samples largely depends on the clinical situation, resources and the local expertise available.

Chapter 9 Concluding remarks

9.1 Limitations of the study

9.1.1 Sampling bias

Due to invasiveness of endoscopy, most *H. pylori* studies including this one, has a sampling bias in that only symptomatic patient with suspected gastric diseases significant enough to warrant upper gastric endoscopy were selected and investigated. In addition, samples were taken only from a small part of the stomach. In this study most of the samples were collected from the gastric antrum because although some of the study subjects allowed research biopsies from different regions of the stomach; but most consented only to a single biopsy which was taken from the antrum. Although the antrum is the most colonised area of the stomach as far as *H. pylori* is concerned, it is possible for a different strain or strains to colonise other parts of the stomach such as corpus and/or fundus. This has potentially affected our aim to look at variation within single host in great detail.

9.1.2 Sample size

Over 400 subjects were routinely investigated for possible gastric disease during the course of this study but only a third consented to join the study. Increasing the sample size could have been more representative, but due to limited resources and invasiveness of

technique, this was not possible. Advances in less invasive techniques such as the use of string test, gastric washings which reflects the whole stomach, and stool antigen test [29] may allow increase in sampling and provide opportunity for large epidemiological studies to study the dynamics of *H. pylori* infection and its transmission by looking at asymptomatic individuals and parents whose children are colonised.

9.1.3 Children strains

We have seen in this study that children carried mainly less virulent strains but the numbers investigated were small which limits our ability to make any significant conclusion. Increased sample size would have provided us the opportunity to evaluate the specific features that contribute to the success (from *H. pylori*'s perspective) of initial colonization, and subsequent evolutionary changes that lead to adaptation, and potentially gastro-duodenal disease after many years of chronic colonization. This limitation was due to lack of adequate resources and the need for an experienced paediatric endoscopist and the related patient care associated with such patients.

9.1.4 Lack of histological assessment

Gastric diseases were mostly diagnosed by macroscopic examination of the stomach only and the classification of diseases may have been inaccurate without histological assessment. In a study to investigate gastric intestinal metaplasia, about 8% with normal gastroduodenal tract appearance by endoscopy may have had premalignant gastric lesions [333]. Thus, histological examination of gastric biopsies could have increased the accuracy of gastro duodenal disease classification.

9.1.5 Culture and mixed infections

H. pylori is a fastidious organism and biopsies are often contaminated with gastric and upper respiratory tract microbiota. Only 53% of gastric biopsies cultured were successfully isolated and purified. This was mainly due to either failure to survive subculture or contamination despite the use of a selective media. In addition, the repeated passaging of cultures to obtain a confluent growth for storage and analyses may have contributed to over enhancement of minority strains and also influence changes in the bacterial genotype due to recombination/mutation thereby over representing the presence of mixed infections in culture as indicated in chapter 5.

9.1.6 Data

The only endoscopy unit in The Gambia is found in MRC, Fajara where the study was conducted. A bias may have arisen on the prevalence of gastric diseases and geographic locality of patients as only select patients are seen in this unit; those who can afford the travel distance and those lucky to obtain endoscopy as only a limited number of endoscopies are undertaken each day. Most patients from other regions outside of GBA stayed with relatives on the coast during the course of investigations and treatment and gave these locations as their addresses instead of where they actually come from. A robust demographic information system and a good follow up will in future be necessary to provide insights into the prevalence of gastric diseases in the different regions and also relatedness of strains and possible sources of infections.

9.1.7 Genotypes and gastric diseases

Several studies have identified many genes that were implicated in the development of gastric diseases [334,335]. At least in Western countries [335], the presence of *homa*, *homb*, *sabA* were also found to be associated with the development of gastric diseases. In a study of Colombian subjects [329] using micro-array, *Jhp0045* and *Jhp0046* were shown to be associated with gastric carcinoma in *cagA* positive

cases. Thus, the detection of only *cagA* and *vacA* in this study is limited in the information that they provide.

9.1.8 Phylogeny of *H. pylori*

MLST is often regarded as the “gold standard” method to determine the genetic relatedness of *H. pylori*. The test produces data that is highly unambiguous, reproducible, and portable and can discriminate between many sequence types and can be used to investigate evolutionary relationships among bacteria. However, a limitation of MLST is the need for DNA sequencing, which is restricted to few institutions. Further, due to the sequence conservation in housekeeping genes, MLST sometimes lacks the discriminatory power to differentiate bacterial strains, which may limit its use in epidemiological investigations. To improve the discriminatory power of MLST, a multi-virulence-locus sequence typing (MVLST) approach has been developed using *Listeria monocytogenes* [336]. MVLST broadens the benefits of MLST but targets virulence genes, which may be more polymorphic than housekeeping genes.

9.2 Future studies

1. Given that more genes than those examined here are implicated in the development of gastric diseases [334,335], it would be useful to investigate the presence of other genes in this largely unstudied population and their relationship with gastro-duodenal diseases.
2. *H. pylori* share their gastric niche with numerous other strains and bacterial species (and some protozoan species) and given that *H. pylori* engages in DNA exchange with other strains and bacterial species, metagenomic analysis would provide us the opportunity to study the composition of the stomach microbiome in relation to presence or absence of *H. pylori* and genes encoded in the different species.
3. DNA sequencing used in this study only looked at highly conserved housekeeping genes; the rest of the genome may be different. About 60 of our strains have been deposited at the genome centre in Washington University, St. Louis, USA for whole genome sequencing.
4. The use of a minimally invasive technique such as the string test to look at asymptomatic subjects, children and parents whose children are colonised could allow us further to evaluate the prevalence of *H. pylori* in The Gambia and possible transmission routes.

9.3 Closing remarks

Despite these limitations, this study provides a detailed initial description of a set of *H. pylori* isolates directly related to a geographically defined West African population. This has answered some relevant questions on *H. pylori* virulent genes in association with gastro-duodenal diseases in this population that may have contributed to the gene pool of *H. pylori* in Europe and the Americas, and described susceptibility and resistance to clinically relevant anti-*H. pylori* agents that are commonly used in The Gambia and reported the molecular basis of their anti-*H. pylori* activities. All of the clinical and virtually the entire laboratory procedures were conducted in The Gambia, providing a comprehensive basis for future studies into areas that need to be explored further and in greater detail.

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Chapter 11 Appendix A – Study approvals

DMID IRB Approval Certification

Purpose: A completed and signed IRB Approval Certification form certifies that the documents listed in section III were submitted and approved by the IRB listed in section II and does not replace a valid IRB approval letter or stamped informed consent form.

COMPLETION INSTRUCTIONS

1. Provide information requested in sections I, II and III.
2. The Site Principal Investigator must review, hand sign and date the completed form.
3. Please read carefully. If any information is incorrect, please contact the PPD-CTM Essential Documents group at email address: dmdiregdoc.distribution@wilm.ppdj.com.
4. Fax the completed, signed and dated form to FAX (919) 654-4665 or send as an attachment to the email address: dmdiregdoc.distribution@wilm.ppdj.com.

1. GENERAL INFORMATION:

DMID Protocol Number: 06-0053

Full Protocol Title: Genotypes of *Helicobacter pylori* in West African Children and Adults:
Part 1: The Development and Evaluation of Minimally Invasive Techniques for Obtaining *H. pylori* Isolates from Adults and Children in The Gambia

Site Principal Investigator: Prof Richard A. Adegbola

II. IRB/IEC INFORMATION: Please complete one form for each IRB/IEC, if applicable.

IRB/IEC Name: The Gambian Government/MRC Ethics Committee

III. **DOCUMENT INFORMATION:** Provide a complete list of the IRB approved documents, including version number and/or date with corresponding approval date. Examples of document types: protocol, protocol amendments and 'protocol-associated' documents, such as the informed consent form, study advertisements, or written information given to study subjects.

Document(s) Submitted	Version Number and/or Version Date	IRB/IEC Approval Date
Protocol Number 06-0053	1, 19 March 2007	April 27 th 2007
Information sheet and Consent form (Adults)	1, 19 March 2007	April 27 th 2007
Information sheet and Consent form (Children)	1, 19 March 2007	April 27 th 2007

I attest that the above documents were submitted and approved by the IRB/IEC(s) listed in section II and that all information, including version numbers/dates and IRB/IEC approval dates, on this form is correct.

PI Signature

27 June 2008
Date

Richard A Adegbola
PI Name (please print)



Scientific Coordinating Committee
Medical Research Council Laboratories Fajara
Tel (220) 4494564/4496715 FAX (220) 4496513/4494498
Email: scc@mrc.gm
Website (intranet): [\\open.mrc.gm\scc\home.asp](http://open.mrc.gm/scc/home.asp)

12th April 2007

Dr Richard Adegbola
MRC
Fajara

Dear Richard

RE: L2007.23 - 19th March 2007. Genotypes of *Helicobacter pylori* in West African children and adults (Ref SCC 988v2): Protocols version 1 of 06-0004 and 06-0053 dated 12th March 2007 and 19th March 2007 respectively.

Thank you for your letter which was considered at the recent SCC meeting on April 7th. As the SCC was happy to approve the current versions of your protocol, namely version 1 (13th March 2007) of DMID protocol number 06-0004 and version 1 (13th March 2007) of DMID protocol number 06-0053, as outlined in your letter. It was also noted that Dr Robert Walton is now performing most of the endoscopies in the hospital and may therefore be added to the list of investigators.

With best wishes

Yours sincerely

A handwritten signature in dark ink, appearing to read 'Sarah', is written below the text 'Yours sincerely'.

Prof. Sarah Rowland-Jones
Chair, Scientific Coordinating Committee



Scientific Coordinating Committee
Medical Research Council Laboratories Fajara
Tel (220) 4494564/4496715 FAX (220) 4496513/4494498
Email: scc@mrc.gm
Website (intranet): open.mrc.gm/scc/home.asp

24th April 2007

Dr Richard Adegbola
MRC
Fajara

Dear Richard

RE: L2007.23 - 19th March 2007. Genotypes of *Helicobacter pylori* in West African children and adults (Ref SCC 988v2): Protocols version 1 of 06-0004 and 06-0053 dated 12th March 2007 and 19th March 2007 respectively.

Thank you for your letter which was considered at the recent SCC meeting on April 7th. In addition to my letter dated 12th April 2007, I am happy to note that the protocol is consistent with the grant.

With best wishes

Yours sincerely

A handwritten signature in dark ink, which appears to read 'Sarah (Rowland-Jones)', is written over a faint, larger version of the same signature.

Prof. Sarah Rowland-Jones
Chair, Scientific Coordinating Committee

The Gambia Government / MRC Laboratories Joint

ETHICS COMMITTEE

c/o MRC Laboratories Fajara
P. O. Box 273, Banjul
The Gambia, West Africa

Fax: +220 - 4494498 or 4496 513
Tel: +220 - 4484 073-9 ext. 409

30th April 2007

Dr Richard Adegbola
Head of Bacterial Diseases Programme
MRC Fajara

Dear Dr. Adegbola

RE: L2007.23 - 19th March 2007. Genotypes of *Helicobacter pylori* in West African children and adults (Ref SCC 988v2): Protocols version 1 of 06-0004 and 06-0053 dated 12th March 2007 and 19th March 2007 respectively.

Thank you for submitting the proposal documents for the above study. These and your covering letter and the SCC's letter were discussed during the Gambia Government/MRC Joint Ethics Committee meeting on April 27th 2007.

Your proposal was approved based on the following submitted documents:

- Protocol Number 06-0004 Version 1, 12 March 2007
- Protocol Number 06-0053 Version 1, 19 March 2007
- Appendix Number 06-0004 Version1, 12 March 2007
- Appendix Number 06-0053 Version 1, 19 March 2007

We also note that Dr Michael Walther is the Safety Monitor for this project. Concern was raised about performing endoscopy in ill malnourished children but it was clarified that this is mostly clinically indicated as an investigation for those with slow recovery after initiation of nutritional rehabilitation.

We would like to review for annual renewal of approval in twelve calendar months.

Best wishes

Yours sincerely


Mr. Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

The Gambia Government / MRC Laboratories Joint Ethics Committee:

Mr Malcolm Clarke, Chairman
Mrs Kathy Hill, Secretary
Mrs Norwah Ali, 2nd Secretary
Professor Ousman Nyan, Scientific Advisor
Mr Davada Jagne
Mrs Bertha Mboge
Mr. Modou Phall

Professor Tumani Corrah
Professor Hilton Whittle
Dr Stephen Howie
Dr Mariatou Jaliow
Dr. Lamin Sidibeh
Mr. Malamin Sonko

Chapter 12 Appendix B – Information sheets and consent forms

Bi –Adult information sheet

DMID Protocol 06-0053 (Part 1) Appendix A: Information Sheets
Version 1.0 19 March 2007
Medical Research Council Laboratories

Genotypes of *Helicobacter pylori* in West African children and adults

Research study information sheet for adults

Your illness is suggestive of a disease that is caused by infection with a bacterial germ called *Helicobacter pylori*. This infection results in damage to the lining of your gut and this leads to the pain that you are experiencing. Your doctor will like to examine the lining of your gut to investigate the cause of your illness by passing a scope into your gut in order to decide which is the appropriate treatment for your illness. This test is called an endoscopy. In addition to your doctor's examination, we would like to study this problem in a research study to help us to develop better ways of caring for people who are infected and are sick. This research project will be studying a germ called *Helicobacter pylori*. The results of our findings will be available for the use of your doctor at the MRC ward.

Infection with this germ can be treated. If you are found to have this infection and signs of disease, you will be given antibiotic treatment that normally kills the germ.

When you have your endoscopy test, the doctor will take some tiny pieces of tissue (about 10 to 14 mg), called biopsies, from your stomach to see if this germ is present. If the germ is detected, the doctor will arrange for you to have appropriate treatment. We will also squirt a small amount of liquid (2-5 mL or a teaspoonful) down the endoscope, with a syringe and suck this back with a syringe to look for the germ. This process will not cause you any extra health risk.

We hope that up to 75 adults and 20 children who have endoscopy tests here at the MRC ward will join this study this year. All information collected as part of this study will remain confidential and can only be seen by scientists and doctors working on the study, the ethical review board and by representatives of the study sponsor.

If we detect the germ in your stomach, then we will store samples for future studies in The Gambia and overseas.

You should not participate in this study if you have a history of serious organ disease or have a history of bleeding problems.

You are free to decide whether or not you will participate in this study or not. If you decide not to participate in this study, this will not affect the standard of clinical care that you would normally receive. Your attending physician will continue to treat you even if you decide not to participate in this study. There will be no compensation for you for participating in the study and it will be done at no extra costs to you.

If the results of the study are written up for publication your identity will remain confidential.

This study is sponsored by USA National Institute of Allergy and Infectious Diseases (NIAID).

The Joint Gambia Government and MRC Ethics Review Board can review the subject records whenever required.

You may ask any questions that you have. Further information can be sought from Dr. Corrah (who is also a member of the Ethics Review Board, and can answer any questions you might have about your rights and the way in which this study is being conducted) and Sr. Vivat Thomas. We hope that you will agree to participate in this study and thank you very much for your cooperation.

I further agree that specimens obtained from me for research purposes may be kept for further study of bacterial infections at some future date.

Do you have any questions?

Contact for further information:

Dr. Tumani Corrah
MRC Laboratories, Fajara
Banjul, The Gambia
Phone (+220) 4495442
Email: tcorrah@mrc.gm

Dr. Richard A. Adegbola
MRC Laboratories, Fajara
Banjul, The Gambia
Phone (+220) 4494491
Email: radegbola@mrc.gm

Sr. Vivat Thomas
MRC Wards, Fajara
P O Box 273, The Gambia, Phone (+220) 4497114

Bii – Adult consent form

Medical Research Council Laboratories

Genotypes of *Helicobacter pylori* in West African children and adults

Consent Form for Adults

The information sheet has been explained to me and I understand it or I have read and understood the information sheet.

I understand what participation in this study means to me.

I understand the information regarding me in the course of this study will remain confidential.

I understand that laboratory tests will be done tissues and fluid that I will provide and that, parts of these will be stored for further tests at Fajara and abroad.

I understand that I am free to take part in this study or refuse and that I can withdraw from the study at any time. If I decide to withdraw from the study I understand that this will not in any way affect the standard of clinical care I would normally receive.

I have asked all the questions that I wanted to ask and they have been answered to my satisfaction.

I further agree that specimens may be kept for further study at some future date

I agree to take part in the study.

Signature or thumbprint of participant: -----

This form has been read by/I have read and explain the form to (write name of participant) in a language that s/he understands. I believe that s/he has understood what I explained and that s/he has freely agreed to participate in the study.

Signature of field worker:-----

Name and code of field worker-----

Date: |__|__|/|__|__|/|__|__|__|__|

I have witnessed that this form has been read and explained to (write name of participant) in a language that s/he understands. I believe that s/he has understood what has been explained and that s/he has freely agreed to participate in the study.

Signature of witness:-----

Name of witness-----

Date: |__|__|/|__|__|/|__|__|__|__|

Biii – Children information sheet

DMID Protocol 06-0053 (Part 1) Appendix A: Information Sheets Version 1.0
19 March 2007

Medical Research Council Laboratories

Genotypes of Helicobacter pylori in West African children and adults

Study participant information sheet

Your child has severe malnutrition and is not growing well as other children. An important cause of this problem is bacterial infections that cause damage to the gut. This causes the child to be sick and not absorb food well. We would like to study this problem to help us develop special treatments that may be necessary to heal the gut and help the child to grow better.

To know this we will need to examine your child's gut for infections. To do this we will look at the gut with a special camera and also take fluid and very small pieces of tissue from the gut lining. Your child will be asleep throughout this examination. This has been done many times before to other children with severe malnutrition and there have been no problems with the procedure.

If your child gets sick at any time, your child can receive medical treatment at the MRC Fajara Ward and the Royal Victoria Teaching Hospital.

You are free to decide whether or not your child will participate in this study. You may ask any questions that you have. Further information can be sought from Dr. Corrah and Sr. Vivat Thomas. We hope that you will agree that your child will participate in this study and thank you very much for your cooperation.

Do you have any questions?

Contact for further information:

Dr. Tumani Corrah
MRC Laboratories, Fajara
Banjul, The Gambia
Phone (+220) 4495442
Email: tcorrah@mrc.gm

Dr. Richard A. Adegbola
MRC Laboratories, Fajara
Banjul, The Gambia
Phone (+220) 4494491
Email: radegbola@mrc.gm

Sr. Vivat Thomas
MRC Wards, Fajara
P O Box 273, The Gambia Phone (+220) 4497114

Biv – Children consent form

Medical Research Council Laboratories

Genotypes of *Helicobacter pylori* in West African children and adults

Consent Form for Parents

The information sheet has been explained to me and I understand it or I have read and understood the information sheet.
I understand what participation in this study means to me and my child.
I understand the information regarding me in the course of this study will remain confidential.
I understand that laboratory tests will be done on tissues and fluid that my child will provide and that, parts of these will be stored for further tests at Fajara and abroad.
I understand that we are free to take part in this study or refuse and that I can withdraw from the study at any time. If I decide to withdraw from the study I understand that this will not in any way affect the standard of clinical care that my child would normally receive.
I have asked all the questions that I wanted to ask and they have been answered to my satisfaction.
I further agree that specimens may be kept for further study at some future date

I agree my child taking part in the study. Name of Child: -----

Signature or thumbprint of parent: -----

This form has been read by/I have read and explain the form to (write name of parent) in a language that s/he understands. I believe that s/he has understood what I explained and that s/he has freely agreed for his/her child to participate in the study.

Signature of field worker:-----

Name and code of field worker-----

Date: |__|__|/|__|__|/|__|__|__|__|

I have witnessed that this form has been read and explained to (write name of parent) in a language that s/he understands. I believe that s/he has understood what has been explained and that s/he has freely agreed for his/her child to participate in the study.

Signature of witness:-----

Name of witness-----

Date: |_|_|/|_|_|/|_|_|_|_|



Human Participant Protections Education for Research T

Completion Certificate

This is to certify that

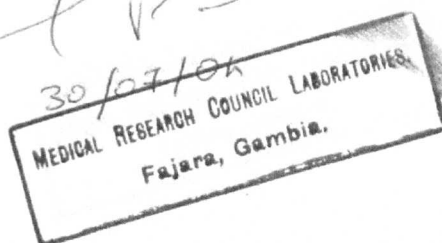
Julian Thomas

has completed the **Human Participants Protection Education for Research Teams** online course, sponsored by the National Institutes of Health (NIH), on 07/29/2004.

This course included the following:

- key historical events and current issues that impact guidelines and legislation on human participant protection in research.
- ethical principles and guidelines that should assist in resolving the ethical issues inherent in the conduct of research with human participants.
- the use of key ethical principles and federal regulations to protect human participants at various stages in the research process.
- a description of guidelines for the protection of special populations in research.
- a definition of informed consent and components necessary for a valid consent.
- a description of the role of the IRB in the research process.
- the roles, responsibilities, and interactions of federal agencies, institutions, and researchers in conducting research with human participants.

National Institutes of Health
<http://www.nih.gov>





Human Participant Protections Education for Research Teams

Completion Certificate

This is to certify that

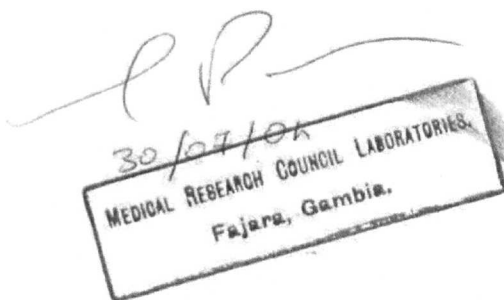
Julian Thomas

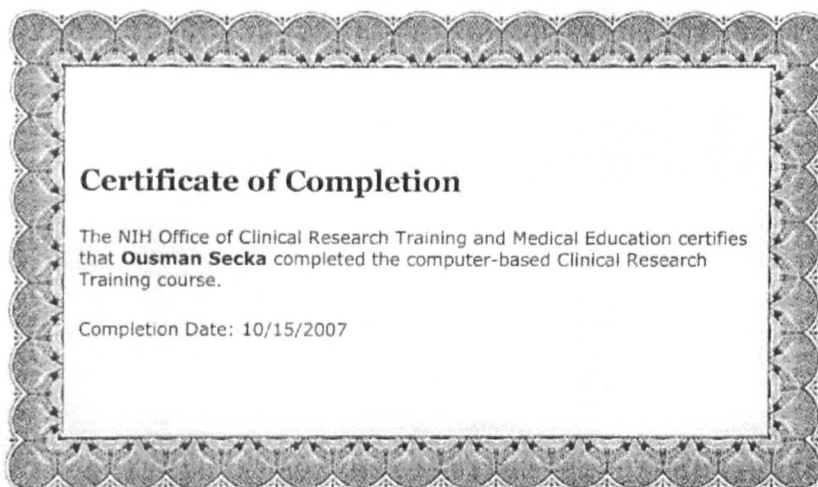
has completed the **Human Participants Protection Education for Research Teams** online course, sponsored by the National Institutes of Health (NIH), on 07/29/2004.

This course included the following:

- key historical events and current issues that impact guidelines and legislation on human participant protection in research.
- ethical principles and guidelines that should assist in resolving the ethical issues inherent in the conduct of research with human participants.
- the use of key ethical principles and federal regulations to protect human participants at various stages in the research process.
- a description of guidelines for the protection of special populations in research.
- a definition of informed consent and components necessary for a valid consent.
- a description of the role of the IRB in the research process.
- the roles, responsibilities, and interactions of federal agencies, institutions, and researchers in conducting research with human participants.

National Institutes of Health
<http://www.nih.gov>





http://www.nihtraining.com/crtpub_508/certify.html?killycache=854352&userID=seck74... 15/10/2007

Certificate of Completion

The NIH Office of Clinical Research Training and Medical Education certifies that **MARY S E TAPGUN** completed the computer-based Clinical Research Training course.

Completion Date: 05/18/2008



Family Health International

certifies that

Hydara Kutub

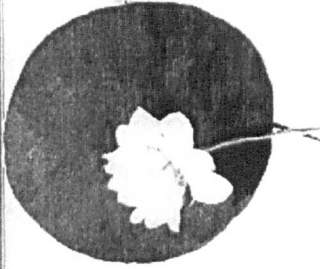
has completed the

RESEARCH ETHICS TRAINING CURRICULUM

January 2008

David Borasky

David Borasky, CIP
Director,
Office of International Research Ethics



TRAINING ON THE PROTECTION OF HUMAN SUBJECTS IN CLINICAL RESEARCH: DMID/PPD- Essential Documents Collection and Submission Process CD package

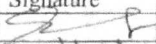
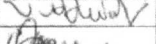

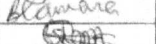
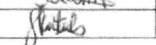


Protocol Number: 06-0053

Protocol title: Genotypes of *Helicobacter pylori* in West African children and adults:
Part I: The development and evaluation of a minimally invasive technique for obtaining *H. pylori* isolates from adults and children in The Gambia

Topics:

- History and Ethics of Clinical Research
- Investigator Responsibilities
- Informed Consent
- Ethics committees

This is to confirm that I have received training on the above topics.

No	Name	Position	Signature	Date
1	Dr. Mary SE Taggun	Physician		17/4/08
2	Sr. Vivat Thomas	Nurse endoscopist		17/4/08
3	Ousman Secka	Study Coordinator		17/04/2008
4	Recard Richards	Nurse		17/04/2008
5	Buba Camara	Nurse		17.04.08
6	Estina Lucy Thomas	Nurse		17.4.08
7	Kutub Hydara	Laboratory Technician		17.04.2008

Name PI: PROFESSOR RICHARD ADEBOLA

Signature PI: 

Date: 17/04/2008

Chapter 14 Appendix D – Media preparations

Di - Preparations of antibiotic stock solutions

1. Trimethoprim*

$$(5\mu\text{g/ml} = 5000\mu\text{g}/1000\text{ml} = 5\text{mg/L})$$

To make 20ml stock solution

- i. $5 \times 20\,000 = 100\,000\mu\text{g}/20\text{ml} = 100\text{mg}/20\text{mls}$
- ii. Therefore $1\text{ml} = \frac{100\,000}{20} \times 1 = 5000\mu\text{g/ml} = 5\text{mg/ml}$ of stock
- iii. 1ml of stock to $1000\text{ml} = 5\mu\text{g/ml}$

2. Vancomycin

$$(6\mu\text{g/ml} = 6000\mu\text{g}/1000\text{ml} = 6\text{mg/L})$$

To make 20ml stock solution

- i. $6 \times 20\,000 = 120\,000\mu\text{g}/20\text{ml} = 120\text{mg}/20\text{mls}$
- ii. Therefore $1\text{ml} = \frac{120\,000}{20} \times 1 = 6000\mu\text{g/ml} = 6\text{mg/ml}$ of stock
- iii. 1ml of stock to $1000\text{ml} = 6\mu\text{g/ml}$

3. Polymixin B

$$(10\mu\text{g/ml} = 10000\mu\text{g}/1000\text{ml} = 10\text{mg/L})$$

To make 20ml stock solution

- i. $10 \times 20\,000 = 200\,000\mu\text{g}/20\text{ml} = 200\text{mg}/20\text{mls}$
- ii. Therefore $1\text{ml} = \frac{200\,000}{20} \times 1 = 10\,000\mu\text{g/ml} = 10\text{mg/ml}$ of stock
- iii. 1ml of stock to $1000\text{ml} = 10\mu\text{g/ml}$

4. Bacitracin

$$(200\mu\text{g/ml} = 200\,000\mu\text{g}/1000\text{ml} = 200\text{mg/L})$$

To make 20ml stock solution

i. $200 \times 20\,000 = 4\,000\,000\mu\text{g}/20\text{ml} = 4\,000\text{mg}/20\text{mls} = 4\text{g}/20\text{ml}$

ii. Therefore $1\text{ml} = \frac{4\,000\,000}{20} \times 1 = 200\,000\mu\text{g}/\text{ml} = 200\text{mg}/\text{ml}$ of stock

iii. 1ml of stock to $1000\text{ml} = 200\mu\text{g}/\text{ml}$

5. Nalidixic acid

$$(10\mu\text{g/ml} = 10\,000\mu\text{g}/1000\text{ml} = 10\text{mg/L})$$

To make 20ml stock solution

i. $10 \times 20\,000 = 200\,000\mu\text{g}/20\text{ml} = 200\text{mg}/20\text{mls}$

ii. Therefore $1\text{ml} = \frac{200\,000}{20} \times 1 = 10\,000\mu\text{g}/\text{ml} = 10\text{mg}/\text{ml}$ of stock

iii. 1ml of stock to $1000\text{ml} = 10\mu\text{g}/\text{ml}$

6. Amphotericin B

$$(8\mu\text{g/ml} = 8\,000\mu\text{g}/1000\text{ml} = 8\text{mg/L})$$

To make 20ml stock solution

i. $8 \times 20\,000 = 160\,000\mu\text{g}/20\text{ml} = 160\text{mg}/20\text{mls}$

ii. Therefore $1\text{ml} = \frac{160\,000}{20} \times 1 = 8\,000\mu\text{g}/\text{ml} = 8\text{mg}/\text{ml}$ of stock

iii. 1ml of stock to $1000\text{ml} = 8\mu\text{g}/\text{ml}$

7. Amoxicillin, Clarithromycin, Erythromycin and Tetracycline

$$(2\mu\text{g/ml} = 2000\mu\text{g}/1000\text{ml} = 2\text{mg/L})$$

To make 20ml stock solution

iv. $2 \times 20\,000 = 40\,000\mu\text{g}/20\text{ml} = 40\text{mg}/20\text{mls}$

v. Therefore $1\text{ml} = \frac{40\,000}{20} \times 1 = 2000\mu\text{g/ml} = 2\text{mg/ml}$ of stock

vi. 1ml of stock to $1000\text{ml} = 2\mu\text{g/ml}$

8. Metronidazole

$$(8\mu\text{g/ml} = 8000\mu\text{g}/1000\text{ml} = 8\text{mg/L})$$

To make 20ml stock solution

vii. $8 \times 20\,000 = 160\,000\mu\text{g}/20\text{ml} = 160\text{mg}/20\text{mls}$

viii. Therefore $1\text{ml} = \frac{160\,000}{20} \times 1 = 8000\mu\text{g/ml} = 8\text{mg/ml}$ of stock

ix. 1ml of stock to $1000\text{ml} = 8\mu\text{g/ml}$

*NB: Trimethoprim is first dissolved in Ethanol

Dii - Ordinary *H. pylori* media for subculturing & antibiotic susceptibility

1. Weigh 39g of Columbia agar (Unipath; Cat no CM331B) powder
2. Suspend in 900mls of distilled water and mix gently to dissolve.
3. Sterilize by autoclaving at 121°C for 15mins
4. Cool to 55°C in cooling water bath
5. Add 20mls of 2% vitox (Unipath; Cat no SR090A) and mix
6. Add 100ml Laked Horse blood (Unipath; cat No SR0048C) and mix.
7. Pour 20mls of media in single compartment petridish and allow to set.
8. Flame, label and store in plastic bag at +4°C until use.

Diii - Selective Columbia blood agar media

1. Weigh 39g of Columbia agar (Unipath; Cat no CM331B) powder
2. Suspend in 900mls of distilled water and mix gently to dissolve.
3. Sterilize by autoclaving at 121°C for 15mins
4. Cool to 55°C in cooling water bath
5. Add 20mls of 2% vitox (Unipath; Cat no SR090A) and mix
6. Add 1ml of the following antibiotics: Trimethoprim (5µg/ml),
Vancomycin (6µg/ml), Polymixin B (10µg/ml), Bacitracin
(200µg/ml), Nalidixic acid (10µg/ml), Amphotericin B (8µg/ml).
7. Add 100ml Laked Horse blood (Unipath; cat No SR0048C) and
mix.
8. Pour 20mls of media in single compartment petri-dish and allow
to set.
9. Flame, label and store in plastic bag at +8°C until use.

Div- Semi-Selective Columbia blood agar media

1. Weigh 39g of Columbia agar (Unipath; Cat no CM331B) powder
2. Suspend in 900mls of distilled water and mix gently to dissolve.
3. Sterilize by autoclaving at 121°C for 15mins
4. Cool to 55°C in cooling water bath
5. Add 20mls of 2% vitox (Unipath; Cat no SR090A) and mix
6. Add 1ml of the following antibiotics: Trimethoprim (5µg/ml),
Vancomycin (6µg/ml), Amphotericin B (8µg/ml).
7. Add 100ml Laked Horse blood (Unipath; cat No SR0048C) and
mix.
8. Pour 20mls of media in single compartment petri-dish and allow
to set.
9. Flame, label and store in plastic bag at +8°C until use.

NB. This media with trimethoprim, vancomycin and amphotericin that do not act against *H. pylori* as described by Akada [253] suppresses possible contaminating bacteria and moulds.

Dv - Glycerol broth (20%)

Procedure

1. Prepare Brain Heart Infusion broth as described.
2. Weigh 47 g of Brain Heart Infusion broth (Unipath; Cat No. CM0225B)
3. Add 1000ml of Distilled water

To prepare 100ml of glycerol broth

1. Brain Heart Infusion Broth..... 80ml
2. Glycerol (Sigma; Cat No. G8773)..... 20ml
3. Mix well and distribute in 5mls amounts in screw-cap tubes or bottles
4. Sterilise by autoclaving at 115°C for 20 minutes
5. This medium should be within the range pH 7.2 - 7.6 at room temperature.
6. Put in the fridge for longer storage.

Dvi - Urea (0.03M solution)

1. Weigh 0.18g of urea (Sigma; cat. No. U6504)
2. Dissolve in 100mls of distilled water
3. Add a small amount of phenol red
4. Sterilize by filtration
5. Aliquot in eppendorf tubes

Store at +4 to +8°C. Solution is stable for 4 weeks.